IDENTIFICATION AND CHARACTERIZATION OF MONOCYTE PROSTAGLANDIN SYNTHASE 2 ACTIVITY AS A RISK FACTOR FOR AND COMPONENT OF HUMAN AUTOIMMUNE DISEASE

By

Sally A. Litherland

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1997

For my parents and family

and in loving memory of

Mrs. Nellie Morgan and Dr. Les Morgan,

my guardian angels and my inspiration.

ACKNOWLEDGMENTS

When a project of this magnitude is undertaken even under normal circumstances, it represents the cooperative effort of many hard working individuals. When done on an accelerated timeline as this one, it would be impossible without a coordinated team effort of experienced experts, dedicated trainees, and talented support personnel. The author wishes to express her heartfelt gratitude to all who contributed to the successful completion of this work.

First and foremost, the author wishes to thank her mentor, Dr. Michael J. Clare-Salzler, who took a chance on the unknown commodity that was this very determined individual, and allowed her to try her own ideas as well as freely share his own with her. Mike is not only a gifted doctor and scientist, but also a truly good human being. The author is honored to call him mentor and friend.

This work would not have been possible without the generous participation of the many men, women, and children who volunteered to donate their time and blood for analysis. The author wishes to express her greatest appreciation and admiration for such selflessness in the name of helping others who may be affected by autoimmune disease.

Next, the author thanks the other members of her committee, Dr. Ammon Peck, Dr. Edward Wakeland, and Dr. Charles Allen, for their consistent support of this doctoral work, their friendship, and sage advice.

Special thanks go to the Flow Cytometry laboratory personnel, Melissa Chen, Neal Benson, and Kristie Grebe, for all the kind help and advice in development of the methodology, data collection, and analysis for the flow cytometric data. Thanks too to the author's laboratory personnel, especially Dr. Tony Xie, whose seminal work in autoimmune mice was the basis and rationale behind this work in humans.

The author also thanks her friends and colleagues for their support and friendship, especially Scott Reno, Kryzsztof Witek, Win Noren, Robbin Chapman and the rest of her SLSTP students and colleagues, Mimi Shao and the Mission Operations Crew, Dr. Lyle Moldawer and his lab group, Rose Mills, Bertha and Javier Sanchez, Donna Whittaker, Ramelle Ruff, Joanne Johnson-Tardieu, Clive Wasserfall, Janet Cornellis, Dr. Jeff Anderson, Roberta Cook, Mary Alice Dennis, and Karen Fuller.

Finally, but most importantly, the author wishes to thank her parents, Allyn and Regina Litherland, for their faith in her, their love, and their unfailing support of her educational endeavors.

TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

IDENTIFICATION AND CHARACTERIZATION OF MONOCYTE PROSTAGLANDIN SYNTHASE 2 ACTIVITY AS A RISK FACTOR FOR AND A COMPONENT OF HUMAN AUTOIMMUNE DISEASE

By

Sally A. Litherland

August, 1997

Chairman: Michael J. Clare-Salzler, MD
Major Department: Pathology, Immunology, and Laboratory Medicine

Recent work in the nonobese diabetic mouse has demonstrated that macrophage produced prostaglandin E2 (PGE2) contributes to antigen presenting cell dysfunction and the development of diabetes. The overproduction of PGE2 was the result of constitutive expression of the normally inducible cyclooxygenase, prostaglandin synthase 2 (PGS2).

In this study, PGS2 expression was examined in over 200 samples of human peripheral blood monocytes from normal controls, relatives of autoimmune patients, and members of a test subject group consisting of autoimmune individuals, diabetics, and individuals at low, moderate and high risk for immune mediated diabetes (IMD). Aberrant PGS2 expression in unactivated peripheral blood monocytes was found in 37% of the test subjects as compared with 4% of normal controls. PGS2 expression correlated

inversely with insulin secretory capacity, a diagnostic measure of risk for diabetes; indicating that aberrant PGS2 expression may predispose individuals to high risk of IMD.

Six of twelve subjects tested had lost sensitivity to interleukin 10 suppression, a normal regulatory control of PGS2 expression. Further investigation is needed to determine if this type of regulatory dysfunction is an underlying mechanism for the defect in vivo.

When PGS2 expression was inhibited in vitro, test subject T cells increased IL2 receptor expression (CD25). The PGE2 suppression of CD25 correlates positively with the IMD susceptibility major histocompatibility locus alleles DR4 and DQβ0302. Through its effect on CD25 PGE2 inhibits interleukin 2(IL2) signal transduction in T cells, a critical signal for T cell activation, proliferation, and activation induced cell death (AICD).

Subject cells were resistant to AICD induced by anti-*FAS* antibody and had an accumulation of ceramide, a second messenger in cell death signal transduction. Ceramide accumulation was directly correlated with the increase in PGS2 expression found in these individuals. Passive cell death, defined as spontaneous death of cells ex vivo, was significantly lower in test subject cells than controls. Concurrently, the percentage of cells remaining in G0/G1 and entering cycle was significantly higher.

This study confirms the identification of aberrant PGS2 expression as a risk factor for human IMD and presents evidence for its role in the immunopathogensis of multiple autoimmune diseases.

CHAPTER 1
EXPERIMENTAL BASIS AND RATIONALE

Autoimmunity

In autoimmune disease, normal regulatory controls for self-tolerance are superseded and the body's immune defenses are turned against its own tissues (Cohen and Young, 1992; Mountz et al., 1994;Ucker et al., 1994). Tolerance of self antigens by peripheral blood mononuclear cells (PBMC) is maintained by regulatory mechanisms including anergy, active suppression by regulatory cells and factors, and elimination of self-reactive cells by Activation Induced Cell Death (AICD)(Squier et al., 1995; Podack, 1995). Though normal healthy individuals can have autoreactive immune cells, these are controlled either by elimination of the cells themselves or functional regulation through a complex system of cell and cytokine interactions. If the control of these cells is compromised, immune mediated pathology can result, leading to overt autoimmune disease (Cohen and Young, 1992; Mountz et al., 1994;Ucker et al., 1994).

Autoimmune Insulin Dependent Diabetes

One of the most common of autoimmune diseases in humans is immune mediated diabetes (IMD), also referred to as insulin dependent diabetes mellitus type1 (IDDM1) or juvenile onset diabetes. This debilitating disease afflicts 1 out of every 5000 Americans and leads to major health complications that are often life threatening. IMD is metabolically characterized as insulin deficiency manifesting as severe blood hyperglycemia and cellular hypoglycemia. Patients diagnosed with IMD must modify dietary intake, self-

monitor blood glucose, and take subcutaneous insulin injections in order to prevent the development of diabetes-related complications including ketoacidosis, retinal disease, kidney disease, neuropathology, and atherosclerosis. Individuals with IMD have a reduced life expectancy by 10 to 20 years as a result of such complications (Winter et al., 1993).

The only confirmed genetic susceptibility locus for IMD is the HLA, major histocompatibility locus (MHC). HLA DR and DQβ loci are linked to both IDDM susceptibility and IDDM resistance. Alleles DR 03 and DR 04 and DQβ 0302 and DQβ 0201 have been described as susceptibility loci; whereas, DR 02 and DQβ 0602 have been defined as protective alleles for IMD (Winter et al., 1993; Faustman, 1993). In addition, approximately 14 non-histocompatibility *Idd* loci linked with IMD susceptibility have been described, though the genes within these loci are largely undefined (Winter et al., 1993).

Individuals genetically predisposed to IMD are further classified with regard to risk by the presence of autoimmune antibodies in their serum. Insulin autoantibodies (IAA+), anti-GAD like domain antibodies (GAD+), and anti- islet cell antigens (ICA+) antibodies can be found in these individuals prior to the manifestation of clinical symptoms. None of these autoantibodies are thought to play a role in the pathology of IMD, but their presence in serum is considered indicative of autoimmunity. Of these autoantibodies, ICA+ antibodies are considered the most predictive for increased risk for IMD (Maclaren et al., 1975).

Unlike many autoimmune diseases, human IMD does not have a clear gender bias in its incidence. However, there is an age effect seen in IMD onset. Higher incidence of disease occurs in pre-pubescent children and adolescents who are at genetic risk for IMD. The peak onset of IMD is found

during puberty and maybe linked to hormonal effects that occur with growth and development(Winter et al., 1993, Leslie and Dubey, 1994).

The Nonobese Diabetic Mouse Model for IMD

Great advances in the understanding of human IMD have come from the genetically inbred mouse model, the nonobese diabetic (NOD) mouse, and congenic strains derived from this line (Serreze et al., 1989; Serreze et al., 1993; Serreze and Leiter, 1988; Serreze and Leiter, 1994; Wicker et al., 1995; Yui et al., 1996). When the mice are housed in a specific pathogen free environment, NOD female mice have an 80% incidence of spontaneous diabetes, while only 20% of males develop diabetes (Serreze and Leiter, 1994). The disease onset is at approximately 5-8 weeks of age. The pathology of the disease in the NOD mice has features in common with IMD in humans, including infiltration of the islets of Langerhans (insulitis), loss of insulin producing islet β cells, MHC involvement, and the development of autoantibodies. The condition is lethal in mice, as in humans, without direct intervention with insulin replacement therapy (Serreze et al., 1989; Serreze et al., 1993; Serreze and Leiter, 1988; Serreze and Leiter, 1994; Wicker et al., 1995; Yui et al., 1996). The NOD mouse strain also develops autoimmune destruction of salivary and lacrimal gland function similar to Sjogren's Disease, and autoimmune thyroid disease (Faveeuw et al., 1994).

Antigen Presentation in Autoimmunity

Antigen presenting cell (APC) activation of T cells is impaired in both animal models and humans with a genetic predisposition for immune mediated diabetes (Lee et al., 1988; Ihm and Yoon, 1990; Clare-Salzler and Mullen, 1992; Clare-Salzler et al., 1992; Clare-Salzler, 1994 and 1995). Defects found in the MHC locus of both NOD mice and humans contribute to APC

impairment (Winter et al., 1993; Serreze et al., 1993; Serreze and Leiter, 1994; Clare-Salzler et al., 1992).

T cell receptor (TCR) stimulation is the primary signal for activation of specific T cells. Antigen presentation by self MHC on APC provide the TCR with a critical stimulus for T cell activation, as can anti-CD3 antibody binding and superantigen crosslinkage of TCR to MHC (Groux et al.,1993, Kawabe and Ochi, 1991). APC transfer experiments in NOD mice have shown a protective effect of dendritic cells against the development of autoimmune diabetes (Clare-Salzler et al., 1992). These findings give evidence that highly stimulatory APC-induced T cell stimulation is essential in the regulation of self- tolerance (Clare-Salzler et al., 1992; Clare-Salzler and Mullen, 1992; Jansen et al., 1994).

Interactions of APC and T cells are crucial to the regulation of the immune response. During maturation in the thymus, stem cell derived precursors of T lymphocytes encounter MHC presented self antigens in its ontogeny. These cells must run a gauntlet of interactions with APC: testing for both negative and positive responsiveness to self. Failure to present the correct level of self-recognition at each stage of this process, that is, MHC recognition for positive selection and low self antigen recognition for negative selection, results in the elimination of such cells before they can be released into the periphery (Jones et al., 1990; Webb et al., 1990).

Not all self antigen-recognizing lymphocytes are eliminated during ontogeny. Autoreactive cells are found in the peripheral blood of both nonautoimmune and autoimmune individuals. In the periphery, qualitative and quantitative differences in the antigen presentation and the microenvironment of the APC-T cell interaction control the outcome of T cell

activation. APC presentation of self-antigens to mature T cells leads to the induction of tolerance; whereas, presentation of foreign peptides can start the T cell activation process necessary for mounting a specific immune response (Ucker et al., 1993, Clare-Salzler, 1995).

Quantitative differences in interaction with antigens during T cell-APC encounters have been implicated as a major component for the production of regulatory T cells as opposed to the production of effector T cells (Ucker et al., 1993). High levels of antigen presentation are thought to lead to production of suppressor/regulatory functional T cells; whereas, lower levels of T cell activation would lead to the development of effector cells (Ucker et al., 1993).

The presence or absence of co-stimulation is critical to how a T cell interprets the primary antigen signal from the APC. Secondary signaling involving B7:CD28 surface molecule interactions allows for full activation of responsive T cells, so that they proliferate and differentiate, and mount an immune response to the antigen. Modulation of the second signal, such as delaying or blocking of the CD28:B7 signal, induces anergy. Cytokine mediated signals, such as IL1, IL4, IFNγ, and IL2, promote T cell differentiation. Variations in the quality and quantity of activation signals also contribute to positive regulation of *FAS* and *FASL* surface molecules, priming the system to eliminate cells and to control or terminate an immune response (Cook and McCormick, 1995).

Activation Induced Cell Death in Autoimmunity

In normal, healthy individuals, selective cell death is used to control the immune response of T cells recognizing self antigens (Martin et al., 1994; Majno and Joris, 1995; Squier, 1995; M. Chen et al., 1995). Selective cell death

is an effective means to maintain control over potentially damaging activation events that could turn the immune system's defenses against self (Squier et al., 1995). This cell death is thought to be mediated by activation induced cell death (AICD). Only cells undergoing high levels of activation are sensitive to AICD, yielding selectivity in the process. Impairment of AICD may be a critical event in the immunopathogensis of autoimmune diseases as it may allow for the accumulation and inappropriate activation of autoreactive T cells (Sneller et al., 1992; Mountz et al., 1994; Thompson, 1995).

When the AICD pathway of a cell is triggered by biochemical signals and events, it leads to the orderly disassembly of the cell's structure into membrane enclosed particles termed apoptotic bodies (Steller, 1995). These particles allow for phagocytic removal the dying cell by surrounding cells or professional phagocytes without concurrent release of intracellular components. The packaging of the cell remnants prevents an inflammatory response from being triggered by release of intracellular antigens and biochemically reactive components; thereby, decreasing the chances of cell damage by a self-directed immune response (Mountz et al., 1994; Steller, 1995).

Elimination of autoimmune T cells through AICD is a mechanism used in the development of peripheral tolerance (Liu and Janeway, 1990). This elimination of self-responsive T cells has been found to be specific for antigen or superantigen activated cells. This specificity of AICD is mediated by the TCR-mediated recognition of the antigen-MHC complex (Webb et al., 1990; Groux et al., 1993).

AICD acts in concert with cell proliferation to control the overall immune response. Like proliferation, AICD of T cells occurs in the setting of

cell activation mediated through the TCR. Superantigen induced activation of the TCR in vivo and in vitro has been used as a model system in which to define the progress of an immune response from induction to termination. In this model system, superantigens such as *Staphylococcus aureus* enterotoxin B (SEB) and retroviral MTV Mls-1a protein, specifically activate a Vβ subclass of T cells (Kawabe and Ochi, 1991;Huang and Crispe, 1993; Gonzalo et al., 1994;Weber et al., 1995; Nishimura et al., 1995). These superantigens first stimulate the T cells of the specific TCR subclass to rapidly proliferate. Fully activated T cells undergo an array of changes including phospholipase A2 (PLA2) induction, reactive oxygen intermediate generation, down regulation of the TCR and other changes in their cell surface markers including upregulation of *FAS* and *FASL* expression (Huang and Crispe, 1993;Weber et al., 1995;Nishimura et al., 1995). The T cell response is rapidly diminished by elimination of the activated T cell population. The expression of *FAS* and *FASL* is a critical for the induction of apoptosis in responding T cells by AICD (Kawabe and Ochi,1991; Dhein et al, 1995). These experiments together suggest that the progression of cells from activation to death is a normal regulatory mechanism for control of the lymphocyte population.

Signaling for Induction of AICD in the Immune System

AICD is an active process, requiring protein synthesis and signal transduction to initiates events within the nucleus of the cell (Steller,1995). It is thought that all cells are capable of undergoing a preprogrammed 'suicide' process (Ucker et al., 1993; Nagata and Golstein, 1995).

In the immune system, the *FAS* receptor/*FASL* ligand binding system is known to induce AICD in the *FAS* bearing cell(Alderson et al, 1993). *FAS/FASL* receptor-ligand proteins have been cloned and studied in humans and mice

(Nagata and Golstein, 1995; Suda et al., 1993; Alderson et al., 1993; Cheng et al., 1995). These proteins are members of the TNFα-TNFαR families of regulatory proteins (Nagata and Golstein, 1995; Owen-Schaub et al., 1992). *FAS* has been found to be homologous to *APO-1* (Cifone et al.,1993; Krammer et al., 1994). *FAS* is a receptor protein expressed on almost all cells of the body, but is either in an inactive state or in low abundance so that it is not readily recognized by the *FASL* bearing cells of the immune system under normal conditions (Nagata and Golstein, 1995). *FASL*, its ligand, is inducible and found on few cell types including monocytes and both CD4+ and CD8+ subpopulations. *FASL* binding of *FAS* is a primary mechanism of cytotoxic T lymphocytes for killing of targeted cells by apoptosis (Owen-Schaub et al., 1992; Podack, 1995). AICD mediated by *FAS/FASL* interactions first requires recognition of antigen through the TCR, activation of the T cell, and cooperation of the target cell for cell death to occur. Triggers for *FASL* induction include TCR-MHC engagement either by antigen or TCR-directed antibody, starvation for growth factors, cell maturation, anergy, and stimulation by TNFα, IFNγ , IL1β,antigen, superantigen, or GM-CSF and CSF-1(Groux et al., 1993; Huang and Crispe, 1993;Wu et al., 1994; Kim et al., 1991; Liu and Janeway, 1990; Kawabe and Ochi, 1991; Brunner et al., 1995). Many of these AICD triggering molecules are also known to stimulate T cell proliferation. The variation in the response to the same factor may be dependent on quantitative variations and factors influencing the cell and its microenvironment (Alderson et al., 1993; Gulbins et al., 1995).

The discovery of two mutant strains of mice, *lpr/lpr* mice and *gld/gld* mice, has suggested the importance of AICD in the immunopathogensis of autoimmune diseases (Sneller et al., 1992; Chervonsky et al., 1997). *Lpr/lpr*

mice are defective in their production of *FAS* receptor; whereas, *gld/gld* mice are deficient in *FASL* (Watanabe-Fukunaga et al., 1992). The *FAS-FASL* signal transduction pathway is considered the primary mechanism for AICD in immune cells. These mice are viable but have high levels of nonmalignant lymphocyte proliferation as well as develop lymphomas and leukemias at an unusually high rate. This increase in both nonmalignant and cancerous immune cell overgrowth in these mice, suggests that the loss of AICD dramatically affects the normal clonal deletion of lymphocytes. In the periphery, proliferating 'undying' self-reactive cells found in these mice increase their potential risk for the development of autoimmunity (Howie et al., 1994). These mouse strains eventually develop characteristic autoimmune responses and disease that parallel those seen in human systemic lupus erthematosus (SLE)(Sneller et al., 1992; Emlen et al., 1994).

Signal Transduction of AICD

Once a cell receives the AICD signal through *FAS-FASL* or *TNFα-TNFαR* binding, the next steps in the process are to transmit the signal via secondary and tertiary messengers to the nucleus of the cell, prompting activation of the death program genes and deactivation of cell proliferation genes(Mountz et al., 1994).

When a cell is signaled to undergo AICD by *FAS/FASL* binding, PLA2 activity is induced within the cell (Jayadev et al., 1994; Schutze et al., 1994; Hannun, 1994; Hannun and Obeid, 1995). The induction of PLA2 in turn catalyzes the turnover of fatty acids and phospholipids in the membrane (Jayadev et al., 1994; Wu et al.,1994; Obeid et al., 1993). As the catabolism of the membrane lipids continues, the cell flips portions of the lipid bilayer of its outer membrane so that phosphoserine containing regions are now visible on

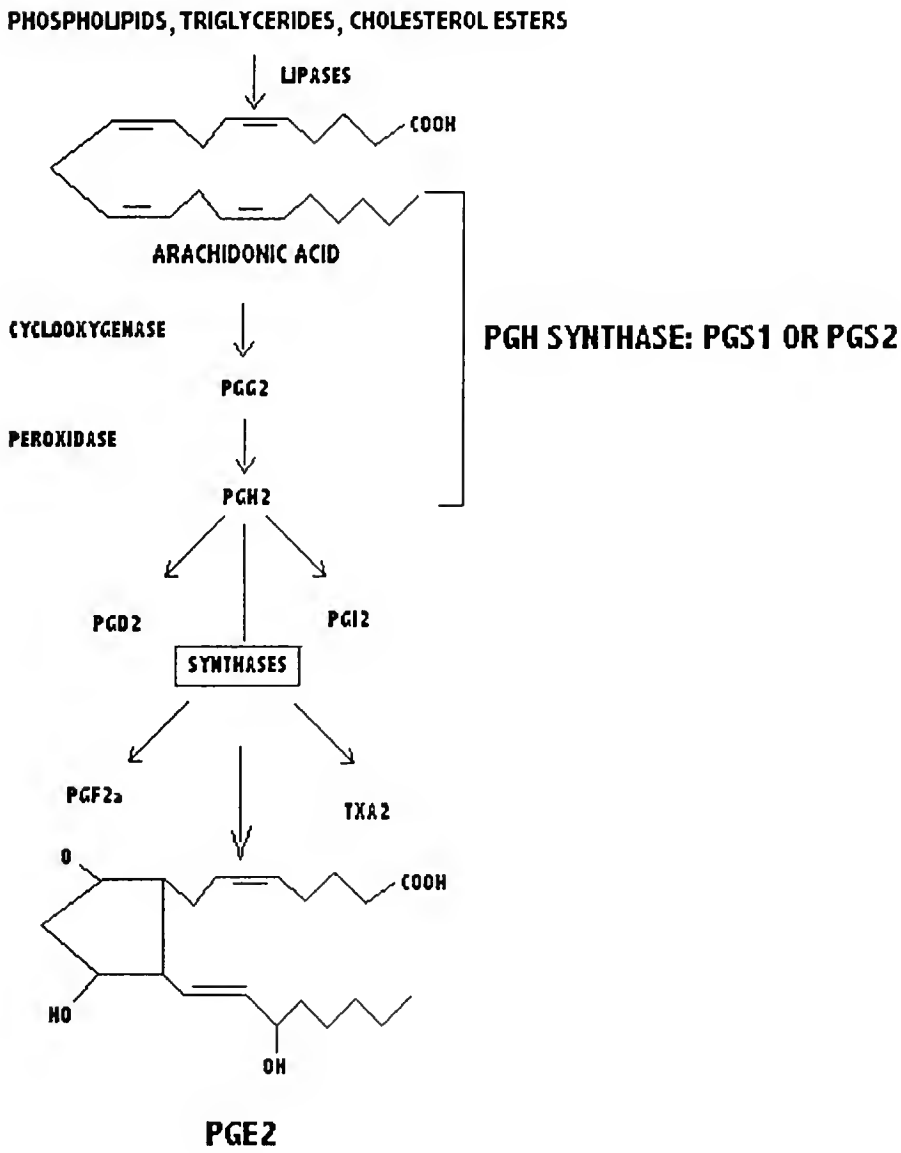
the outer leaf of the membrane (Steller, 1995). This abnormal lipid arrangement is recognized by receptors on phagocytic cells and causes the removal of the dying cell even before it completes the AICD process (Hannun and Obeid, 1995). Activation of lipid metabolism in the membrane also triggers activation of the enzyme sphingomyelinase within the cell which in turn cleaves sphingomyelin from the cell membrane into fatty acids and ceramide (Cifone et al., 1993). The actions of ceramide effectively turn off expression of genes that drives the cell through its cycle and induce others that are involved in the progression of the AICD (Kinoshita et al., 1995; Hannun, 1997; Gill et al., 1994). Ceramide acts a signal transduction molecule in AICD as well as blocking cell cycle and cell survival functions through its effects on *BCL2* and *Rb* gene product function (Hannun, 1994; Hannun and Obeid, 1995;Bose et al., 1995; Hannun, 1997). Ceramide-activated kinases and phosphatases are involved in the activation of chromosomal degradation enzymes that digest the chromatin of the cell into discrete, nucleosomal size fragments (approx.180 bp)(McConkey et al., 1994; Martin et al., 1994; Tian et al., 1995). This uniform or 'ladder' of chromatin fragmentation is considered diagnostic for differentiating apoptotic cell death from oncotic processes of necrosis (Majno and Joris, 1995). In cell death triggered by daunorubicin, a chemotherapeutic agent, it has been shown that even sphingomyelinase-independent production of ceramide allows for the propagation of the AICD signal (Bose et al., 1995).

Prostaglandins and Their Roles in Immune Responsiveness

Prostaglandins (PG) are lipid metabolytes derived from the free fatty acid, arachidonic acid (AA)(Hyslop and Nucci, 1993; DeWitt and Smith, 1995). The prostaglandin family of molecules is important in regulation of

inflammatory responses. Prostaglandin E2 (PGE2), the predominant species produced by blood monocytes and macrophages, is an immunomodulating molecule with potent effects on T lymphocyte activation and function (Figure 1; DeWitt and Smith, 1995).

Figure 1. Biosynthesis Pathways for Prostaglandins. Adapted from Dewitt and Smith, 1995.



Synthesis of Prostaglandins

PG synthesis begins with the release of AA from cell membrane lipids through the actions of phospholipases (PL)(Hyslop and Nucci,1993). AA is converted to PGH2 through the cyclooxygenase and peroxidase activities of prostaglandin synthase, PGS. This enzyme is found in 2 isoforms, PGS1(COX1) or PGS2(COX2). Further conversion of PGH2 to the other PG species, including PGE2, requires additional enzymes that are cell specific in their expression. The prostaglandin synthase step of the pathway is rate-limiting and the focus of regulatory mechanisms controlling prostaglandin production (DeWitt and Smith, 1995).

PGS1 is a developmentally regulated enzyme that promotes and mediates so-called 'housekeeping' functions of circulating hormones in a wide variety of cell types. Its expression is constitutive and dependent on the availability of AA substrate from the actions of cytoplasmic membrane bound PLA2. PGS1 is located in the endoplasmic reticulum membrane and its product acts in both autocrine and paracrine fashions (Morita et al.,1995: DeWitt and Smith, 1995). Homozygous transgenic PGS1 knockout mice are viable and have no apparent pathology if born from heterozygote mating, suggesting that the functions of this enzyme after parturition are not unique and can be replaced by other activities (Langenbach et al., 1995).

PGS2, on the other hand, appears to have essential functions in kidney development, regulation of inflammation and immune response (Morham et al., 1995). PGS2 is bound to the nuclear and nearby endoplasmic reticulum

membranes(Morita et al.,1995). It derives its substrate pool from the induction of cPLA2, a cytoplasmic enzyme that releases AA from cellular lipids in response to activation stimuli (Hyslop and Nucci, 1993). With the cloning and sequencing of both genes, it was found that PGS2 is structurally related, but unique, when compared to PGS1 (Goppelt-Struebe, 1995). The PGS2 gene resides on the distal arm of chromosome 1 in both the human and murine genome (Kosaka et al., 1994). This enzyme is expressed in only a limited number of tissues; mostly as an induced activity during immune and inflammatory responses (Riese et al.,1994). A low basal level of PGS2 expression is found in the immunoprivileged sites, brain and testes, as well as a unique and essential expression found in the macula densa of the kidney (DeWitt and Smith, 1995). Transgenic PGS2 knockout mice develop poorly, and die within 6 weeks after birth, usually due to kidney malfunction and nephritis. These mice also exhibit suppressed immune responsiveness including poor macrophage responsiveness to endotoxin (LPS) stimulus (Sneller et al., 1992; Morham et al., 1995; Tsujii and DuBois, 1995;Chervonsky et al., 1997).

Monocyte/Macrophage Prostaglandin Synthase 2 Expression

Peripheral blood monocytes and tissue macrophages are the major sources of PGE2 within the human immune system. Its expression is considered a hallmark of monocyte and macrophage activation (Sweet and Hume, 1996). During an immune response, PGE2 production is enhanced by the induction of PGS2 expression. Monocyte activation by IL1 α & β, TNF α, or

LPS promotes PGS2 expression within the 6 hours of stimulation (Sweet and Hume,1996; Ristimaki et al.,1994 ; Reddy and Herschmann, 1994). When PGS2 expression is accompanied by a cPLA2 catalyzed release of AA, its activity promotes the production of PGE2 (Dewitt and Smith, 1995).

Regulation of PGS2 expression is done through a complex series of signal cascades involving multiple points of feedback regulation by its product, PGE2. TNFα and IL1 α & β stimulation of monocytes induces PGS2 expression as well as IL12 production. IL12 in turn promotes even higher PGS2 expression. IL12 also promotes IL10 production from monocytes and other immune cells (Segal et al.,1997; Gerosa et al., 1996; Daftarian et al.,1996; Ludviksson et al., 1997; Ehrhardt et al., 1997). IL10 acts as a major regulatory suppressive signal for immune response effects including IL12, TNFα, IL1 α & β, and PGS2 expression as well as the induction of cell surface receptors for secondary activation signals. (de Waal Malefyt et al., 1991; Isomaki et al., 1996; Mertz et al.,1994; Spittler et al.,1995; Meisel et al., 1996; Dai et al., 1997; Takenaka et al., 1997). IL10 suppresses PGS2 expression approximately 16-18 hours after activation (D'Andrea et al., 1993; Strassmann et al., 1994; Niiro et al.,1995; Berger et al., 1996). Therefore, PGS2 expression is self-limiting; in that its product, PGE2, downregulates IL12, TNFα, and IL-1, promoters of PGS2 expression, and upregulates IL10, a suppressor of PGS2 (van der Pouw Kraan et al., 1995; Ludviksson et al., 1997).

Prostaglandin Effects on Immune Microenvironment

PGE2 is a potent modulator of the inflammatory response and is present in abundance during the early response phase of inflammation. Prostaglandins have a physiological role in pain, fever, vasodilatation leading to localized swelling and heat, and phagocytic activity enhancement for activated macrophages and natural killer cells present at wound sites (Lu et al., 1995; DeWitt and Smith, 1995).

Some of same signals that promote PGS2 expression and PGE2 production also promote AICD. These include IL1 α & β, TNFα, IFNγ, and AA (Watson and Wijelath, 1990; Kim et al., 1991; Cifone et al., 1993; Obeid et al., 1993; Jayadev et al., 1994; Ristimaki et al., 1994; Wu et al., 1994). Ceramide, along with its role as a signal transduction molecule in AICD, also promotes the production of PGE2 through induction of PGS2. In turn, PGE2 suppresses the signal transduction activities of ceramide (Ballou et al., 1992; Hannun, 1997). The mechanisms involved in this feedback regulation intertwining these two signal pathways are still poorly understood (Ballou et al., 1992; Jarvis et al., 1994a and 1994b; Hannun, 1994; Hannun and Obeid, 1995).

Many of PGE2 effects on cells are mediated through cAMP (Foegh, 1988;Holter et al., 1991; Snijdewint et al., 1993). Changes in cellular levels of cAMP affects protein kinases involved in cell activation and proliferation control (Anastasia et al., 1992; Paliogianni et al., 1993; Riese et al., 1994; van der Pouw Kraan et al., 1995). PGE2 elevated cAMP levels affects *RAS*

dependent gene expression including *Raf* and *ras* p21 expression (Pastor et al., 1995; Cook and McCormick, 1993; Gulbins et al., 1995). These genes are involved in the activation of MAP kinases which are crucial to the activation of cell proliferation gene expression as well as signal transcription in AICD (Baixeras et al., 1994; Goetzl et al., 1996).

Prostaglandin E2 and T cell Responses

Prostaglandins are potent modulators of T cell activation. PGE2 effects on T cell regulation appear to be two-fold: 1) modulation T cell function and 2) inhibition of AICD signal transduction (Lenardo, 1991; Lu et al., 1995). PGE2 role in cell death shares mechanistic components with its role in the control of cell activation.

Prostaglandin E2 effects on IL2 signal transduction

PGE2 induced cAMP inhibits T cell expression of IL2 and its receptor (IL2R) (Minakuchi et al., 1990; Anastassiou et al., 1992; Paliogianni et al., 1993; D. Chen et al., 1994). By blocking IL2 production and IL2 signal transduction, prostaglandins effectively inhibits T cell proliferation and block their progression to AICD.

PGE2 specifically inhibits the upregulation of the alpha subunit (α, CD25) of IL2R (Antonaci et al., 1991; Giordano et al., 1993). This subunit is expressed on T cells after antigen stimulus and is considered a marker for T cell activation (Schorle et al., 1991; Antonaci et al., 1991; Giordano et al., 1993). The β and γ subunits of the IL2R are constitutively expressed as a low

affinity receptor on mature resting T cells and promote IL2 signal transduction leading to cell proliferation. The signal from this low affinity receptor promotes *BCL2* and *c-myc* driven cell functions that preserve cell viability and drive cell cycling (Ahmed et al., 1997). The α subunit of IL2R is upregulated by T cell activation and complexes with the βγ chains to create a high affinity receptor for IL2. The new level of signal transduction promoted by the α chain containing receptor promotes AICD of T cells. In vitro studies using differential inhibitors of IL2 and non-IL2 dependent cell proliferation showed that this effect is independent of its augmentation of T cell proliferation (Lenardo, 1991; Groux et al., 1993; Miethke et al., 1994; Kishimoto et al., 1995; Sakaguchi et al., 1995; Taguchi and Takahashi, 1996; Kneitz et al., 1995; Fournel et al., 1996; Wang et al. 1996; Parijs et al., 1997; Ahmed et al. 1997; Sharfe et al., 1997; Zhu and Anasetti, 1995). Studies of lymphoid cells from a patient with a novel human immune disorder suggest that loss of IL2R α chain function can also block normal activation induced downregulation of the cell viability factor *BCL-2*; thereby, enhancing the sensitivity of activated T cells to AICD (Sharfe et al., 1997).

Prostaglandin E2 and Metalloproteinase Activity

Another possible mechanism by which PGE2 affects T cell activation and AICD is through prostanoid activation of metalloproteinases, which can enzymatically process surface regulatory molecules such as *FASL*, *FAS*, and TNFαR from cells (Mariani et al., 1995; Kayagaki et al., 1995). Metalloproteinases secreted by monocytes and macrophages during

extracellular matrix remodeling are readily induced by the autocrine activity of PGE2 (Lang and Bishop, 1993; Sunderkotter et al., 1994; Mertz et al.,1994; Clare-Salzler, 1994).

Metalloproteinase release of *FASL* and TNFα has been postulated to upregulate *FAS-FASL* mediated AICD and TNFα mediated cell activation, respectively. However, similar metalloproteinase cleavage of *FAS*, TNFα receptor, and other surface receptors could prohibit signal transduction through these molecules; thereby, leaving T cells resistant to cell death (Goetzl et al., 1996).

PGE2 Role in Diabetes in the NOD Mouse

Recent work in the NOD mouse has revealed that an excess of macrophage produced PGE2 contributes to APC dysfunction(Prescott and White, 1996). The overexpression of PGE2 was found to be the result of a defect in cyclooxygenase expression. Messenger RNA (mRNA) for prostaglandin synthase 2 (PGS2), normally inducible, is expressed constitutively in all NOD macrophages; and PGS2 protein is elevated in the estrus phase of mature females, the most susceptible NOD group for diabetes (Xie, dissertation, 1997).

High levels of PGS2 mRNA have been found in the unactivated macrophages of pre-diabetic NOD mice beginning at 4 weeks of age. NOD macrophages produce elevated levels of PGE2 when cultured alone, when compared with control mouse strains. In addition, in vitro studies have shown

that macrophages from NOD mice have marked elevation in their PGE2 production when co-cultured with NOD T cells (Xie, dissertation, 1997).

The aberrant expression of PGS2 and PGE2 production are corrected by the congenic replacement of the chromosomal locus containing the NOD PGS2 gene with the normal gene locus from a nonautoimmune strain, C57B10. This genetic change is related to a 50% reduction in disease incidence (Wicker et al., 1995; Xie, dissertation, 1997). The reverse congenic; that is, a normal, nonautoimmune mouse (C57B6 background) containing only the chromosome 1 locus (including the NOD PGS2 gene) from the NOD, expresses PGS-2 constitutively and shows lymphocyte infiltration into the pancreas (Yui et al., 1996; Xie ,dissertation, 1997; Garchon et al. 1994).

Confirmation of PGS2 enzymatic activity as the cause of the PGE2 elevated levels seen in the NOD was established by in vitro and in vivo testing of the effects on PGE2 production by drugs that specifically block PGS1 or PGS2 (i.e., indomethacin, for both PGS1 and PGS2, and NS398, specific for PGS2). When applied to T cell-macrophage-coculture systems, these enzyme inhibitors reversed PGE2 production to baseline levels (Futaki et al., 1994; Xie, dissertation, 1997).

Production of high levels of PGE2 by NOD mouse macrophages interferes with AICD induction in Vβ8+ T cells stimulated in vivo and in vitro by SEB superantigen. In vivo and in vitro treatment of NOD monocytes with PGS2 inhibitory drugs reversed this interference and allowed for elimination

of the superantigen stimulated subpopulation of Vβ8+ T cells (Webb et al., 1990; Kawabe and Ochi, 1991; Xie, dissertation, 1997). These data indicate that the PGS2 defect seen in these autoimmune mice has a direct effect on AICD.

Levels of both *FAS*, and its ligand, *FASL* expression are abnormally high in NOD spleen and lymph node cells as compared to nonautoimmune control strains, B6 and BALB/c, in the resting state and with stimulation by anti-CD3 and SEB stimulation TCR activation. These results suggest that elimination of activated T cells by AICD is impaired in NOD mice at a point distal in the signal cascade to these receptors.

Lipid analysis of NOD lymph node cells and the NOD monocyte derived cell line, ZK7, showed higher levels of ceramide, sphingomyelin, and PGE2 in these cells than in lymph node cells of control B6 mice. These high levels of lipids in lymph node cells and monocytes derived from NOD mice are apparent in the unmodified state as well as with TNFα stimulation, as compared with the low but detectable levels of these lipids in the lymph node cells of B6 nonautoimmune mice. The accumulation of ceramide in NOD cells suggests that there is a block in the AICD process distal to ceramide production in the signal transduction pathway.

These data suggest that signal induction for AICD in the NOD is activated, but that the process is blocked later in the signal transduction pathway. One hypothesis is that NOD cells are primed and ready for AICD,

but are somehow restrained from completing the process. Knowing that PGE2 production is aberrantly high in these cells, it is possible that ceramide signal transduction may be blocked at the level of *RAS* activation by the effects of cAMP generated by PGE2 (Gulbins et al., 1995).

IL10 Role in NOD Diabetes

The role of IL10 in NOD diabetes has been the focus of recent studies using NOD transgenic mice. IL10 accelerates onset of autoimmune diabetes in these transgenic autoimmune mouse models. Overexpression of IL10 in the pancreas can replace all *Idd* susceptibility loci except the NOD MHC in promotion of diabetes. IL10 immunosuppression of PGS2, IL12, TNFα, and IL1 in normal immune responses may be impaired in this transgenic model; allowing autoimmune responsiveness to be enhanced rather than suppressed (Lee et al., 1996). The mechanism of this IL10 effect has yet to be elucidated.

PGE2 in Human Autoimmune Disease

Macrophage derived PGE2 has been implicated in the dysregulation of T cell activation characteristic of many autoimmune diseases (Clare-Salzler, 1994 and 1995). PGE2 may influence the generation of effector T cells as opposed to regulatory T cells by altering the quantitative signal the T cell receives via IL2/IL2R binding, IFNγ generation, kinase activation, and *RAS* dependent gene activation (Anastasia et al., 1992; Cook and McCormick, 1993; Paliogianni et al., 1993; Ucker et al., 1993; Riese et al., 1994; van der Pouw Kraan et al., 1995; Mauel et al., 1995). PGE2 roles in blocking AICD are

less well defined. PGE2 effects on cell activation and cell death are most likely not separate and independent mechanisms, as they both appear to be dependent on specific activation signaling between monocytes/ macrophages and T cells as well as overlap mechanistically (i.e., through cAMP elevation and effects on signal transduction).

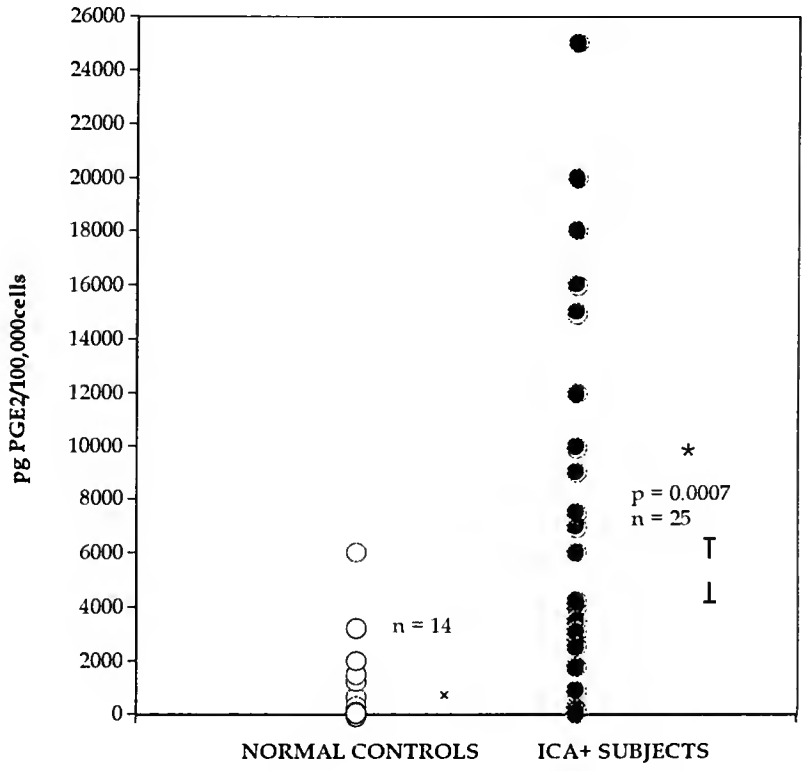
Preliminary Data on PGS2 Expression in Human Autoimmune Diseases

Elevated PGE2 production and concurrent elevation of PGS2 mRNA have been found in cultured peripheral blood monocytes of pre-diabetic individuals, patients with IMD (Figure 2), new onset SLE patients (5/7 tested), and autoimmune thyroiditis patients (4/6 tested). In contrast, PGE2 levels and inducible PGS2 activity were significantly lower in normal controls tested under the same conditions (Clare-Salzler, 1994). These data suggest that the defect in PGS2 expression may be a factor common to a number of autoimmune diseases.

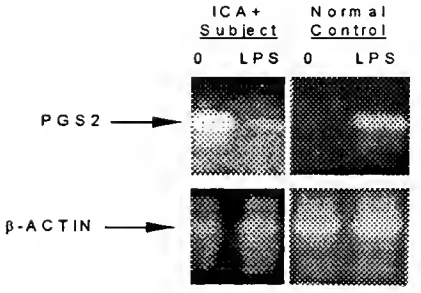
This study focused the potential of spontaneous PGS2 expression and aberrant PGE2 production to have a role in the immunopathogensis of human IMD and other autoimmune diseases. Mechanisms by which a defect in PGS2 expression could manifest, affect cell activation and/or AICD, and correlate with onset of clinical diabetes were characterized.

Figure 2. a. PGE2 Production from Cultured Human Monocytes. b. RT-PCR amplification products from reaction with PGS2 specific primers. Lanes represent gene expression after 24 hr culturing under the following conditions: lane 1) induction with LPS; 2) no induction. G3PDH RT-PCR run in parallel as internal control. c. Summary of PGS-2 expression in autoimmune patients and normal controls. PGS2 mRNA measured by RT-PCR amplification from 100,000 purified monocytes cultured for 16hr. *significant difference ($p<0.0001$) **($p<0.0008$) by Fisher T Test (from Clare-Salzler, 1994).

A.



B.



C.

Subject Group	PGS2 mRNA+/total	% PGS2 mRNA+
Healthy Controls	2/23	8.6%
ICA+/pre-IDD	29/35	83%
ICA-/Established IDD	6/7	85.7%

CHAPTER 2
MATERIALS AND METHODS

Human Subject Populations

Sixty test subjects (aged 3 to 75, 41 females and 26 males) were drawn from volunteers participating in the Clinical Research Center Diabetes Prevention Trial and associated clinical research protocols. All subjects included in the test group were considered autoimmune by virtue of testing positive for autoantibodies or by other clinical criteria. Subject volunteers were drawn from two protocol groups within the test population: 1) Natural History Group (NH) and 2) Subcutaneous Group (SQ).

The NH group contained individuals who were relatives of diabetics and currently not on any trial treatment protocol. These individuals were being monitored their progression toward possible diabetes. These individuals were classified as at lower risk for onset of diabetes by virtue of their intravenous glucose tolerance test (IVGTT) values above the established threshold for their age group; i.e., <100 for >11yrs of age; <75 for children younger than 11, and/or lack of specific islet cell antigen (ICA) antibodies. As individuals in the NH group developed ICA autoantibodies, they were reclassified as being at moderate risk for IMD.

The SQ group consisted of persons considered at high risk for early onset of IMD by virtue of clinical criteria (i.e., IVGTT values below the established threshold for their age group, the presence of antibodies to islet cell antigens (ICA+), and genetic profile (*Idd* risk groups)). These individuals were on a prophylactic protocol of daily subcutaneous insulin injections and blood glucose monitoring. These subjects would stop their treatments at least 3 days prior to clinic visits; and therefore, were not on insulin treatment at the time of sampling.

Subjects with active autoimmune disease (AI) including Hashimoto's thyroiditis, Addison's disease, Graves' disease, vitiligo, ulcerative colitis, and rheumatoid arthritis were found in both NH and SQ groups. In addition, some subjects from both groups developed IMD over the course of the trial and were re-classified as diabetic (D). Subjects were sampled twice on average, with at least 3 months time between samplings.

Ninety control samples were drawn from 30 healthy laboratory or clinic personnel (age 18 to 55, 13 female, 12 male) and from volunteer relatives of autoimmune patients that had with no clinical history of autoimmune disease (age 35 to 45, 1 female and 4 male).

Sample Preparation

Over 200 blood cell samples were drawn following informed consent from autoimmune subjects or their legal guardians and normal controls according to protocols approved for the University of Florida Shands Teaching Hospital IRB. All human blood samples used in these studies were obtained by trained clinical staff and with informed consent as prescribed by the Clinical Research Center. Proper biohazard handling and disposal procedures were used in all work with these samples. Samples were received for analysis within 1 hour of collection and were placed into sodium azide

containing buffer within 90 minute after receipt. A minimum of volume 1ml of whole blood was sufficient for the analysis.

Materials

Endotoxin-free Ficoll-Hypaque was purchased from Sigma and used in a 2/3 ratio to isolate PBMC from whole blood. Phosphate buffered saline (1xPBS) stock was made from endotoxin-free 10x solution (Sigma, Gibco, Whittaker Biochemical) and pH adjusted to 7.4 with sodium hydroxide (Sigma). Gibco RPMI 1640 plus glutamine powdered medium was reconstituted in milli-Q water and supplemented with 2g/L sodium bicarbonate (Baker reagent grade), 10%(v/v) heat-inactivated endotoxin-free fetal bovine serum (HyClone certified grade), and 1%(v/v) PSN antibiotic mix (Sigma penicillin, streptomycin, neomycin mix for tissue culture), pH adjusted to 7.4, then filter sterilized. A solution of 0.01%(w/v) trypan blue (Sigma) in 1xPBS was used for counting viable cells. DAPI chromatin stain (used at 1ug/ml) was a gift from Dr. Michael Paddy of the Center for Structural Biology, University of Florida. Propidium iodide (PI), Lipopolysaccharide (LPS, endotoxin; working concentration of 1ug/ml) and Phytohemagglutanin (PHA, lectin; working concentration of 5-10ug/ml), were purchased from Sigma. The metalloproteinase inhibitor, Galardin, TNFα, and TNFα binding protein were gifts from Dr. Lyle L. Moldawer, Department of Surgery, College of Medicine, University of Florida. Annexin-PI labeling kits were purchased from R-D Research and used according to manufacturer's suggestions.

PCR primers for cell markers and cytokines were the generous gifts of Dr. Ammon Peck and Dr. Jeff Anderson, Department of Pathology, College of Medicine, University of Florida.

ELISA kits for PGE2 were purchased from Cayman Chemical Company and used according to manufacturer's directions. ELISA kits for T cell

cytokines IL2, IL10, and IFNγ from Genzyme and run according to manufacturer's protocols. IL10 ELISA were either purchased from Genzyme or were run as a collaborative effort in the laboratory of Dr. Lyle Moldawer, Department of Surgery, College of Medicine, University of Florida.

Antibodies

Fluorescein (FITC) labeled anti-human PGS2 mouse monoclonal antibody (IgG1) was derived from ascites fluid was a gift from or purchased from the Cayman Chemical Company. This monoclonal antibody was originally developed by Cremion and coworkers (1995) of France and is currently available as a FITC conjugated ascites isolate IgG1 from Cayman Chemical Company. Earlier runs of the flow cytometric (FACS) analysis were performed using an unlabeled rabbit polyclonal anti-PGS2 sera and unlabeled mouse monoclonal antibody, both purchased from Cayman Chemical Company. Mouse monoclonal antibody conjugates (PE or FITC) raised against human monocyte markers, CD14 (IgG2a), CD69 (IgG1), DR (IgG2b), CD80 (IgG1), CD86 (IgG1), TNFα (IgG1), IL10 (IgG2a), CD25 (IgG1), CD4 (IgG1), CD8 (IgG1), and FAS (IgG1) were purchased from Pharmingen. Anti-human CD105 direct FITC label mouse monoclonal antibody (IgM) was a gift of Dr. M. Schieder of Germany, originally purchased from Serotec. Human blood antigen absorbed mouse isotype control antibodies for each of the above antibodies were purchased from Sigma and Pharmingen to serve as nonspecific binding controls for background set point. Non-immune rabbit serum from Cayman Chemical Company was used as the control for polyclonal anti-PGS2 antibody studies. Unlabeled rabbit polyclonal or mouse monoclonal antibodies were detected by goat $(Fab)_2$ FITC-conjugated fragments, specific for rabbit (Sigma) or mouse IgG (Cappel).

Reagents for Label Preparation

Lyophilized mixed mouse serum (Sigma) was reconstituted in endotoxin-free water (Gibco) to a concentration of 1mg protein/ml and used as a blocking agent at a working concentration of 20ug/million cells. Blocking was supplemented with 10ul/100ul cell suspension of autologous human plasma when available. FACS buffer consisted of 1%(w/v) RIA grade BSA(Sigma) and 0.1%(w/v) sodium azide (Sigma) dissolved in 1xPBS and bought to pH 7.4 before filter sterilization. The cells were fixed in a solution of 4% formaldehyde in 1xPBS. Saponin buffer was made by dissolving 0.5%(w/v) saponin permeabilizing agent(Sigma) in FACS Buffer and readjusting the pH to 7.4 prior to filter sterilization. All FACS solutions were stored refrigerated until use.

Cell Preparation

One to twenty milliliters(ml) of whole blood were collected into heparinized vacutainers at each sample drawing. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation (500xg, 30min, 20C) on Ficoll gradients. After the serum layer was removed and sampled, the PBMC were collected from the top of the gradient, washed with 1xPBS, and resuspended in RPMI + 10% FCS. The PBMC were then counted, viability assessed, and diluted to 0.5million cells/200ul with azide containing FACS buffer. To generate a positive controls for PGS2 expression and monocyte activation, aliquots of control cells were transferred to polypropylene culture tubes prior to the dilution in FACS buffer and cultured with 1-10ug/ml LPS , known to readily induces PGS2, (Sweet and Hume, 1996) or with 5-10ug/ml PHA for nonspecific cell activation, for16-24hr at 37°C/5%CO_2 before analysis.

Ex vivo Analysis of PGS2 Expression by Intracellular Flow Cytometry

Previous work on human monocyte PGS2 expression was done with adherence purified monocytes. Macrophage adherence to a surface can induce early response genes such as *c-fos* (Sweet and Hume, 1996). Since PGS2 is considered a marker for monocyte/macrophage activation, a sensitive method for detecting PGS2 without monocyte isolation from freshly isolated PBMC was devised. This method allowed for differentiation of the amount of PGS2 protein expression inherent in subject and control PBMC from that induced by the adherence. In addition, use of the intracellular flow cytometric (FACS) analysis method to detect intracellular PGS2 protein allowed for concurrent detection of defined markers of monocyte activation.

The human PGS2 peptide used to raised the anti-PGS2 monoclonal antibody was kindly provided Cayman Chemical for specificity testing. A *FASL* peptide purchased from Calbiochem was used as a nonspecific antigen control. Monocyte activators, LPS (working concentration of 1ug/ml) and PHA (working concentration of 10ug/ml) , were purchased from Sigma.

Fluorescent Antibody Labeling of Cells for FACS Analysis

All antibodies were used at an optimal working concentration of 0.5-1ug/million cells. Ficoll-isolated PBMC were aliquoted into Falcon 5042 polystyrene tubes at 0.5million cells/tube as dilutions in FACS buffer. The cells were then incubated with 10ul of mouse serum and 10ul of autologous human plasma for 20min at room temperature. After 20min, anti-surface marker antibodies or their isotype controls were added to the appropriate tubes and the cell incubated for an additional 20min at room temperature. The cells were then washed (500xg centrifugation, 15C, 5min) with 1ml of FACS Buffer and the supernatants discarded. 500ul of cell fixation solution was then added to each tube and the cells allowed to fix for 20min. After trial

runs with alternative fixation methods (i.e., paraformaldehyde, ethanol), 4% formaldehyde was found to be the most reproducible and stable fixative which allows retention of surface antigens and stable cell for permeabilization. Fixed cells were washed twice with 500ul of the permeabilizing saponin buffer and the supernatant poured off. Saponin permeabilization was found to be superior to ethanol or Triton/Tween 20 in that it allowed for good surface antigen retention as well as optimal access of antibodies to the target protein. This procedure requires saponin to remain in all solutions post fixation to maintain cell permeability. After the final washes of the labeled cells, the samples are returned to the non-saponin containing buffer to close the pores formed in the membrane. In the residual volume (approximately 200ul), 0.5ug of anti-PGS2-FITC antibody or isotype control was added into the appropriate tubes and allowed to incubate with the permeabilized cells for 1 hour. Following incubation, all tubes were washed 3 times with 500ul of saponin buffer and finally resuspended in 200ul of FACS Buffer. Sample analysis of10,000 events were used each sample on a Becton Dickinson FACSort instrument with an argon laser excitation of 488nm, 15millivolt for FITC and PE, and detected by fluorescence at 530 +/-15 and 580 +/-21 nm, respectively.

For each sample, aliquots of unlabeled cells, and isotype antibodies were run in parallel. In the majority of experiments, sets of subject and control PBMC samples were processed and analyzed in parallel. Analysis of the FACS data was performed using the Becton Dickinson PC LYSYS II program or WinMDI freeware. Positivity for a given antigen was determined as the percentage of cells with a fluorescence intensity above the maximum level of the non-specific (isotype) antibody control as well as by mean fluorescence intensity.

PGS2 specificity testing was done by FACS analysis with antibody preparations that were incubated refrigerated overnight in a 10:1 peptide to antibody ratio of either PGS2 peptide or a nonspecific peptide from *FAS-L* protein as the intracellular labeling material.

This FACS method is highly reliable even with high variance human peripheral blood samples (PBMC) when run with a full complement of controls and standardized to the nonspecific isotype control for antibody background binding. Reproducible and well separated responses to the intracellular labeling of PGS2 in human peripheral blood were obtained by this method (Figure 3). Unactivated normal control PBMC CD14+ cells exhibit low labeling for PGS2 often equal to or lower than the fluorescent labeling seen in the control isotype matched antibody (Figure 3, panel A). Overnight culture in RPMI + 10% FCS yields a low level expression in normal controls (Figure 3,panel B). With 10ug/ml LPS activation, the induction of PGS2 expression was clearly seen as a 1-2 log shift in the fluorescent labeling by PGS2 (Figure 3, panel C).

Specificity/accuracy of the PGS2 expression detection method was tested by pre-absorbing the anti-PGS2 specific monoclonal against either the peptide used in the production of anti-PGS2 antibodies or a nonspecific peptide from *FASL* protein. Over 90% of the anti-PGS2 activity was retained after preincubation with the *FAS-L* peptide; whereas, less than 2% of the activity above the isotype control background remained after incubation with the specific PGS2 peptide (Figure 4).

Multiple, independent samplings from healthy control individuals have low within-sample variation as compared to the population variance (SD=5 to

Figure 3. Contour Plot of Intracellular FACS Analysis of Human PBMC. A.) cells ex vivo without stimulation or culture; B.) cells from the same sample cultured 24hours without stimulation; and C.) cells from the sample cultured 24hours in the presence of LPS.

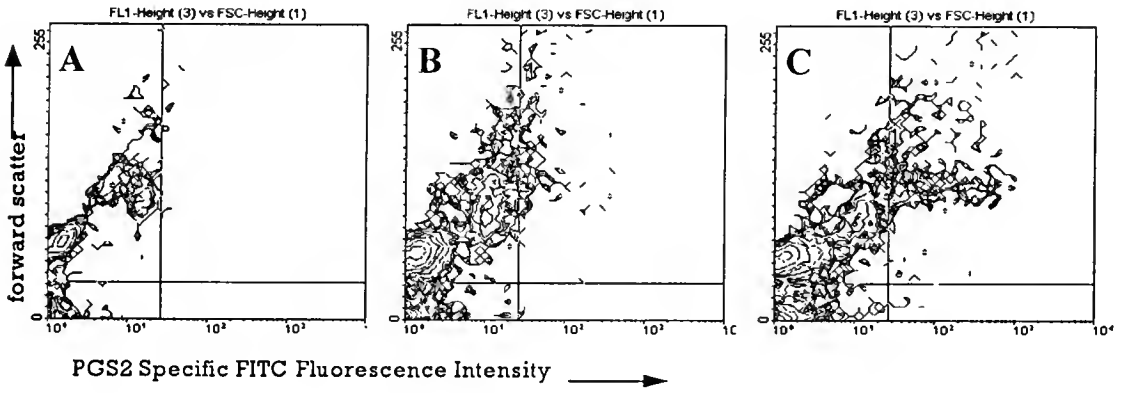
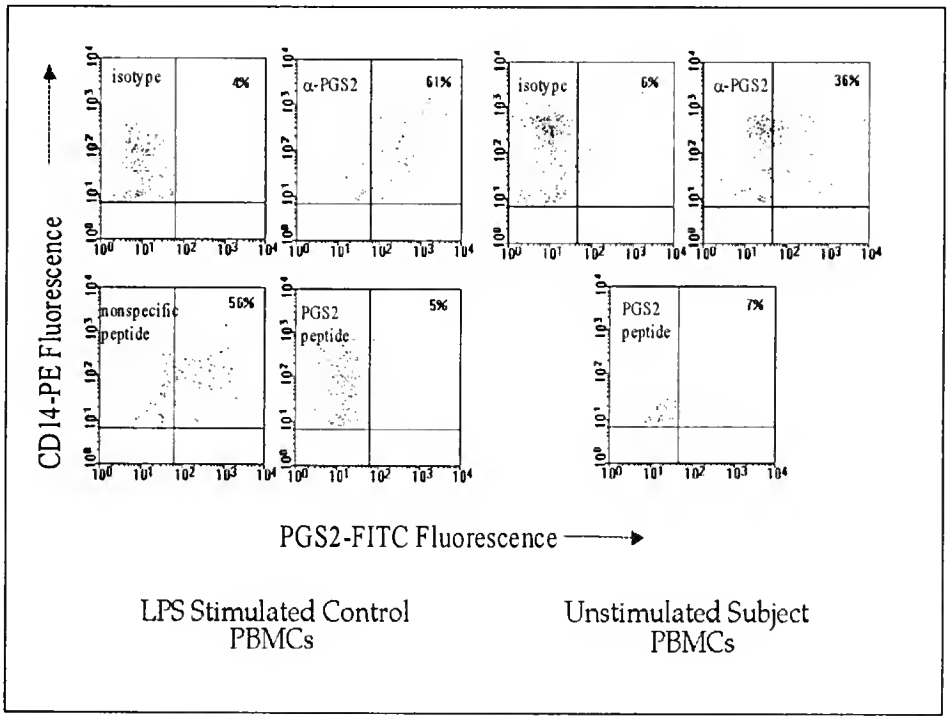


Figure 4. Specificity of Antibodies for Detection of PGS2. LPS stimulated control PBMC (left four dot plots) and unstimulated test subject PBMC (right three dot plots) were subjected to FACS analysis for PGS2 expression. In the upper left corner plot, the samples were labeled with nonspecific isotype antibodies (background fluorescence); the upper right plot cells were labeled with the specific anti-PGS2 antibodies, the lower left plot cells labeled with the anti-PGS2 antibody that had been previously incubated with a nonspecific peptide and the lower right/center plot cells labeled with the anti-PGS2 antibody that had been previously incubated with the specific PGS2 peptide.



7% within sampling; 5% standard derivation for population). Comparison of the sensitivity of this method with that of Western blot analysis using the same antibody for detection of PGS2 shows the FACS method is many fold more sensitive and requires 10-100 fold fewer cells to obtain a detectable signal (Towbin et al., 1979). FACS based analysis is theoretically capable of detecting specific antigen expression down to the single cell level. Dual labeling with the intracellular protein specific antibody in conjunction with cell surface markers can enhance the assay by also defining the population expressing this intracellular antigen. By comparison, Western blot analysis is limited to the level of protein detectable on PAGE gels (at best 10ng by silver staining methods), which requires 100-1000 fold more cells for extraction (Towbin et al, 1979). This would put the quantitative potential of FACS analysis at a level similar to RT-PCR quantitation of mRNA levels in a cell.

Monocyte Activation Analysis by Flow Cytometry

Co-labeling cells for surface markers and intracellular PGS2 can expand the FACS method described above expanded to help identify the cell populations expressing the intracellular PGS2, and help define their activation state. Co-localization studies of two intracellular proteins in the same cell, that is, intracellular TNFα, intracellular IL10 in conjunction with intracellular PGS2 labeling, were also possible and used as further indicators of PBMC activation status (Sweet and Hume, 1996).

Fluorescent Antibody Labeling of Cells for FACS Analysis

All antibodies were used at an optimal working concentration of 1ug/million cells. The cells were then incubated in FACS Buffer with 10ul of mouse serum and 10ul of autologous human plasma for 20 minutes at room temperature(rt). After 20 minutes, anti-surface marker antibodies or their isotype controls were added to the appropriate tubes and the cell incubated

for an additional 20 minutes at rt. The cells were then washed (500xg centrifugation, 15C, 5min) with 1ml of FACS Buffer and the supernatants discarded. Five hundred microliters of Cell Fixation Solution was then added to each tube and the cells fixed for 20 minutes, rt. Fixed cells were washed twice with 500ul of the permeabilizing Saponin Buffer. An 0.5ug aliquot of anti-PGS2-FITC antibody or isotype control was added into the appropriate tubes of permeabilized cells and incubated for 1 hour, rt. Following incubation, all tubes were washed 3 times with 500ul of Saponin Buffer and finally resuspended in 200ul of FACS Buffer. FACS analysis was performed as described above for intracellular FACS. Positivity for a given antigen was determined as the percentage of cells with a fluorescence intensity above the maximum level of the non-specific (isotype) antibody control as well as by mean fluorescence per cell.

In situ Immunohistochemical Analysis of PGS2 and Cellular Markers

Fluorescent labeling of cells was confirmed by microscopic inspection of cytospin preparations made from the FACS cell preparations. DAPI was used to stain chromatin for ease in cell localization under the fluorescent microscope. The same staining method was applied to chamber-well slides of adherent cell cultures for in situ immunohistochemical detection of CD14+/PGS2+ monocytes. For in situ and cytospin labeled cells, post-fixation and permeabilization staining of cellular chromatin with DAPI was used to help locate and quantitate the percent positive cells per field. Photomicroscopy was done on an Olympus IMT-2 Fluorescent Inverted microscopy with the kind permission of Dr. Michael Paddy, Center for Structural Biology, College of Medicine, University of Florida.

RT-PCR Analysis for PGS2, FAS, and Cytokine Profiles

Freshly isolated PBMC samples were collected and aliquots of 1-8 million cells were frozen under 100% ethanol and stored at -80C until analyzed. Frozen cell samples were thawed, centrifuged out of ethanol, and the cell pellet air or vacuum-dried. mRNA was extracted from the cell pellet (approximately 1,000,000 cells) using the QIAGEN rapid mRNA kit method and immediately converted into cDNA by reverse transcription (RT) using Perkin-Elmer reagents and kit protocols. The resultant cDNA was then used as template for PCR amplification using primers specific for human PGS2 gene and either Perkin-Elmer or Qiagen PCR reagent kits. Sample were routinely processed without a hot start for 35 cycles in a GeneMachine II thermocycler using the temperature protocol of 90°C, 105sec, 35 cycles of (72°C 45sec, 65°C 30sec) with a 7-10min extension period at 75°C at the end of the run. Samples were loaded onto 1.0% Agarose gels containing 0.02% ethidium bromide (EtBr, Sigma) and run on IBI electrophoresis system for 1hr at 80V-100V, then visualized and photodocumented using a Stragagene EagleEye transillumination system. Similar analyses were carried out using primers for IL10, IL2, IL4, TNFα, TGFβ, IL12, IL1α & β, FAS, FASL, CD4, CD8, CD25,and CD3.

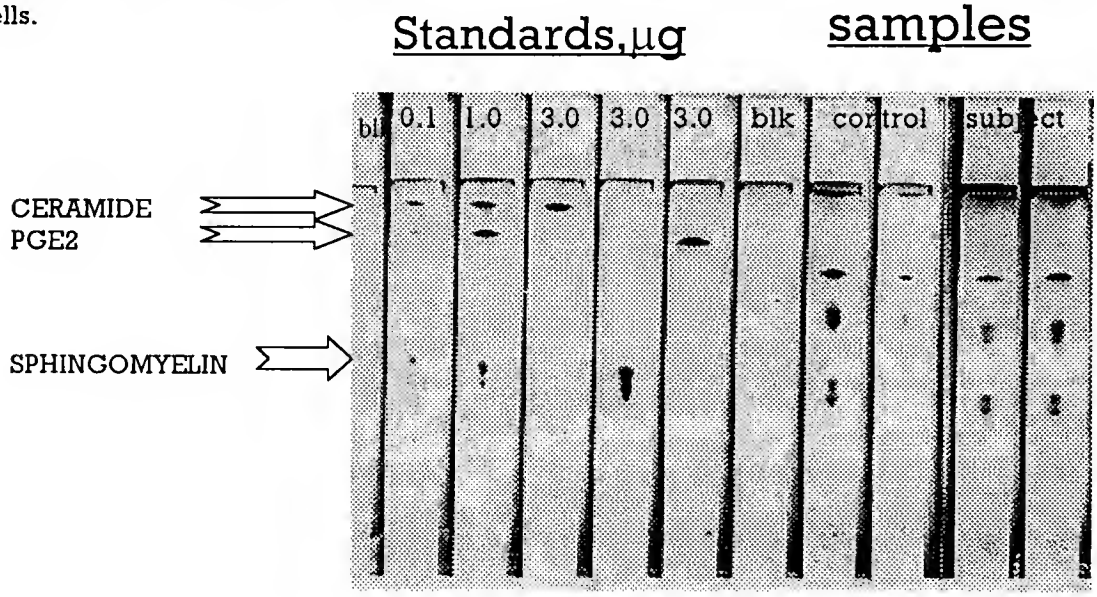
Ceramide Analysis

Cells freshly collected from ficoll separated peripheral blood were extracted for lipids by the method of Bligh and Dyer, 1959. These lipid extracts were stored as dried pellets under nitrogen gas at -80C until analysis. The lipid profile shown in Figure 5 was obtained by separation using High Performance Thin Layer Chromatography (HPTLC) using lipid extracts obtained from 2million cells solubilized in 10-20ul of chloroform just prior to application. The mobile phase used for the majority of analysis was

Chloroform: Methanol: Water, in a volume ratio of 65:25:4. All reagents were HPLC grade and all glassware was alkali and alcohol washed between runs. Lipids were detected by exposure to iodine vapors and analysis was by densitometry scanning (Perkin Elmer MD1 software) relative to known concentration single standards for ceramide, sphingomyelin, and prostaglandin run on the same plate (Figure 5). All data points represent the average of two replicates of each sample as compared to 4 replicates of each standard.

Confirmation of ceramide identification and quantitation was established by a series of HPTLC runs of replicates of the same sample sets (control and subject) in multiple solvent mixes to show comigration of the standard and sample spots. In addition, cell samples were spiked with a known quantity of standard(s) prior to extraction and followed through HPTLC analysis to calculate recovery percentages.

Figure 5. High Performance Thin Layer Chromatography of PBMC Lipids. Example HPTLC plate showing ceramide, PGE2, and sphingomyelin standards along with human PBMC samples from a normal control and an autoimmune subject. Mobile phase is Chloroform: Methanol: Water in a 65:25:4 volume ratio. Sample spots contain lipid extract from 2million cells.



Passive Cell Death, Cell Cycle Effects, and AICD Induction Analyses

To measure the effect of aberrant PGS2 activity in subjects on susceptibility to cell death, passive cell death, progress through cell cycle, and sensitivity to AICD, samples were analyzed using specialized FACS labeling that detected changes in the cell chromatin content and morphological studies for characteristic cellular changes of apoptotic death.

Propidium Iodide Intercalation for Chromatin Content and Cell Cycle Analysis

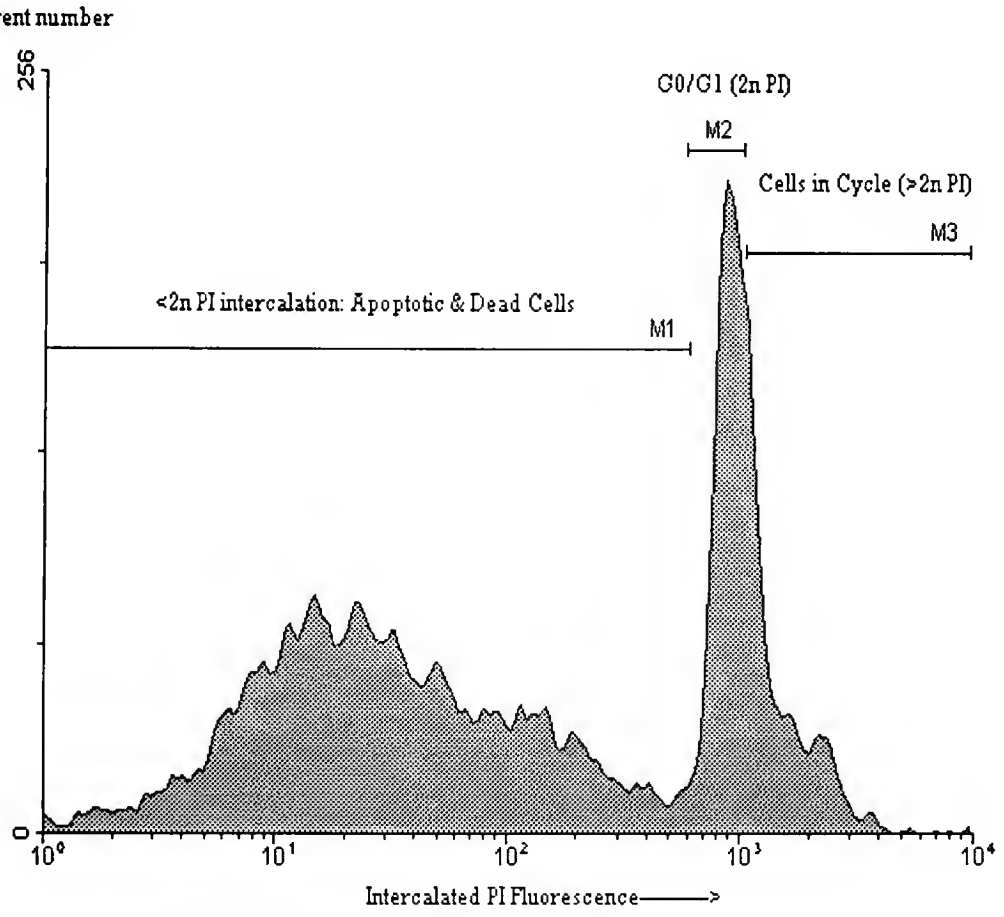
Propidium Iodide (PI) intercalation is detectable by FACS and is proportional to the length and topological structure of cellular chromatin. From one PI intercalation measurement of chromatin content, cell cycle status can be determined. Cells with a full complement of chromatin (2n chromosome number) will define the peak of cells in G0/G1 phase. Those replicating (>2n to 4n) will fluoresce at a greater intensity depicting the S, M, and G2 cycling cells. Cells with less than complete chromatin content (<2n) are in the process of chromatin breakdown and a portion represent cells undergoing apoptotic death (Figure 6).

PBMC were prepared for intracellular FACS as described above with and without surface identification marker labels. At least 30 minutes prior to FACS analysis, 100mg/ml PI in FACS Buffer was added to an aliquot of fixed permeabilized cells and analyzed as is.

Annexin/PI Analysis for Induction of AICD

In an attempt to measure the early events of AICD, freshly isolated PBMC were placed in FACS buffer and 10ul of Annexin and 10ul of PI added to each nonpermeabilized aliquot. These samples are read by FACS analysis within 15minutes to detect cells that can take up the PI (i.e., membrane

Figure 6. Example of Propidium Iodide Intercalation FACS Analysis for Cell Chromatin Content. Histogram depiction of PI intercalation fluorescence shows peaks at less than 2n, 2n, and greater than 2n levels of chromatin indicating cells that have broken chromatin (dead or apoptotically dying), cells with a full compliment of chromatin (2n, cells in G0 and G1 phases of the cell cycle), and cells with greater than 2n chromatin (cells with replicating DNA and dividing cells:G2, S, M phases of the cell cycle).



compromised, dead cells) and surface label with Annexin which binds phosphoserine residues that have flipped to the outer leaf of the cell membrane; an early event in apoptosis.

TUNEL Analysis for AICD

Uniform nucleosome sized cleavage of cellular DNA is considered one of the characteristics of cells undergoing AICD (Mountz et al., 1994; Gold et al., 1994). To analyze this property both qualitatively and quantitatively,

Terminal Transferase tailing with FITC-conjugated UTP was used both in FACS analysis and in situ immunohistochemical protocols. Boeringer-Mannheim In Situ Death Detection Kit reagents were used in cell suspensions or on adherence cell cultures prepared in chamberwell slides. Duplicate cell samples were fixed and permeabilized as described above for intracellular FACS analysis and then mixed with a reaction cocktail containing FITC-conjugated UTP. One sample was left as is and Terminal Transferase (TdT) enzyme was added to the other. The samples were incubated for 1 hour at 37C and then cytospun on to slides for in situ analysis. The samples without TdT acted as background controls for the enzymatically labeled duplicate. TUNEL labeled cells were also run as colabeled samples with intracellular labels for chromatin (DAPI) and surface cell identification markers. FACS analysis for TUNEL labeling was done using the procedures described above for intracellular FACS analysis.

Morphological Analysis for AICD

Cytospins of cells were prepared and stained by Difquik staining for morphology and photodocumentation of the cellular changes of the cells undergoing AICD or PCD: membrane blebbing and nuclear fragmentation with condensed chromatin (Martin et al,1994).

PGS2 Activation and Inhibition Assays

PGS2 activity was both stimulated and suppressed in vitro to test its effects on monocyte and T cell activation. PBMC isolated on ficoll gradients were aliquoted into polypropylene tubes and supplemented with PHA or LPS for nonspecific activation stimulation of T cells and monocytes, respectively. In addition to the stimulatory compounds, cultures were supplemented with inhibitory substances: 10μg/ml IL10 or the PGS2 specific inhibitor, 5μM NS398 or 10μg/ml Galardin, a broad spectrum metalloproteinase inhibitor. IL10 was

used as a physiologically important and appropriate regulator of PGS2 activity; whereas, NS398 was used as a pharmaceutical agent specific for inhibition of PGS2 activity which does not affect gene or protein expression. Galardin was used to counteract the effect of PGE2 induction of metalloproteinases; a potential mechanism of action for PGE2 effects on cell activation and inhibition of AICD. After incubation at 37C/5%CO2 for up to 24 hours, both cell and supernatant samples were taken from these cultures for FACS analysis for PGS2, T cell surface markers, and monocyte activation markers, ELISA analysis of PGE2 and T cell cytokines, and lipid analysis by HPTLC.

Measurement of T cell Activation in PBMC Cultures

Loss of T cell tolerance regulation is central to the current theories for the underlining cellular dysfunction that precipitates autoimmune disease (Katz et al, 1995). To investigate the effect of aberrant PGS2 activity on T cell function and APC-T cell interactions, control and AI PBMC cultures were subjected to T cell activation stimuli. These polypropylene tube cultures were made from ficoll separated PBMC in medium alone or supplemented with 5-10ug/ml PHA or 1-10ug/ml LPS; and with or without the specific PGS2 inhibitor 5μM NS398. The cultures were maintained in 37C/5%CO2 for up to 72hrs before collection and analysis.

T cell Activation Analysis

T cell activation was assessed by FACS analysis for T cell markers CD4, CD8, CD3 and CD25 as well as the monocyte markers described above. Supernatants from these cultures were collected for ELISA determination of T cell cytokines IL2, IL4, IFNγ, IL10, and for PGE2. The fold change in CD25 expression and cytokine production between PHA or LPS alone and PHA or

LPS plus NS398 was used to determine the change in T cell activation with and without PGS2 activity.

T cell Proliferation Analysis

PBMC cultures were established in triplicate in 96 well dishes after ficoll preparation. These cultures were supplemented with 5ug/ml PHA for nonspecific T cell activation with or without concurrent supplementation with 5μM the PGS2 specific inhibitor, NS398. The cultures were allowed to incubate for 3 days at 37C/5% CO2 before 1μCi/well of tritiated thymidine was added to each well. The cells were incubated for 24hrs more and then the suspension cells harvested by suction onto glass fiber filters and extensively washed. After drying, the filters were read by dry count beta counter and analyzed for 3H thymidine incorporation as a measure of proliferation.

T cell AICD Induction Analysis

In order to determine the susceptibility of the PBMC T cell population to AICD induced by PHA, cultures of control and subject PBMC were established in polypropylene tubes as above, in the presence or absence of PHA and with or without concurrent supplementation with NS398 to block PGS2 activity. These cultures were maintained for 1 to 5 days at 37C/5%CO2 then applied to a second ficoll gradient to remove dead cells. The ficoll-repurified cells were plated in 24-well dishes that had been previously coated with and without 2ug/well anti-*FAS*, low endotoxin, azide-free monoclonal antibody. The cells were allowed to incubate 18-22 hours with the antibody at 37C, 5%CO2. The cells were harvested and labeled as described above for T cell markers, TUNEL, and PI intercalation.

Analysis of Hormonal Influence and Menstrual Cycle Effects on PGS2 Expression in PBMC

Previous studies on human and animal uterus and vagina tissues have shown a cyclic expression of PGS2 linked to the female estrus cycle (Leslie and Dubai, 1994). PGS2 expression is elevated in the preluteal phase of the cycle and appears to be involved in control of AICD in uterine lining prior to exfoliation and menstruation.

PGS2 expression in PBMC was tested on blood samples from two healthy female controls, one non-autoimmune and one a relative of autoimmune, both of which were not on estrogen/progesterone therapy. These volunteers reported their cycle day as days from the first day of their last period and were drawn at least once a week from that date through to their next period. These cells from samples prepared for PGS2 FACS analysis and serum samples taken for hormone analysis. It was not possible to collect weekly samples from the test subject individuals since they were available for sampling only once every 3-6 months. However, female subjects and controls were asked to estimate their cycle day at the time of each draw and asked whether or not they were on any hormone therapy at the time of their visits. This information was used to compare cycle timeframe with their PGS2 expression.

In addition, PBMC from both male and female subjects and controls were cultured in polypropylene tubes with and without 1-10ug/ml progesterone or estrogen. These cultures were allowed to incubate 24hr at 37C/5%CO2 and then collected for analysis by FACS.

FACS analysis for PGS2 expression was carried out on the two types of samples as described above. Estradiol RIA analysis on serum samples was

performed by the Department of Pharmacology/Medical Chemistry, College of Pharmacy, University of Florida laboratories.

Statistical Methods for Data Analysis

Data from the above analyses was analyzed for statistical significance using Student T test for pair wise comparisons, ANOVA for multifactoral correlative analysis and nonparametric analysis for high variance comparisons. Microsoft Excel, Instat, GraphPad Prizm, Cricket Graph III software was utilized in these analyses.

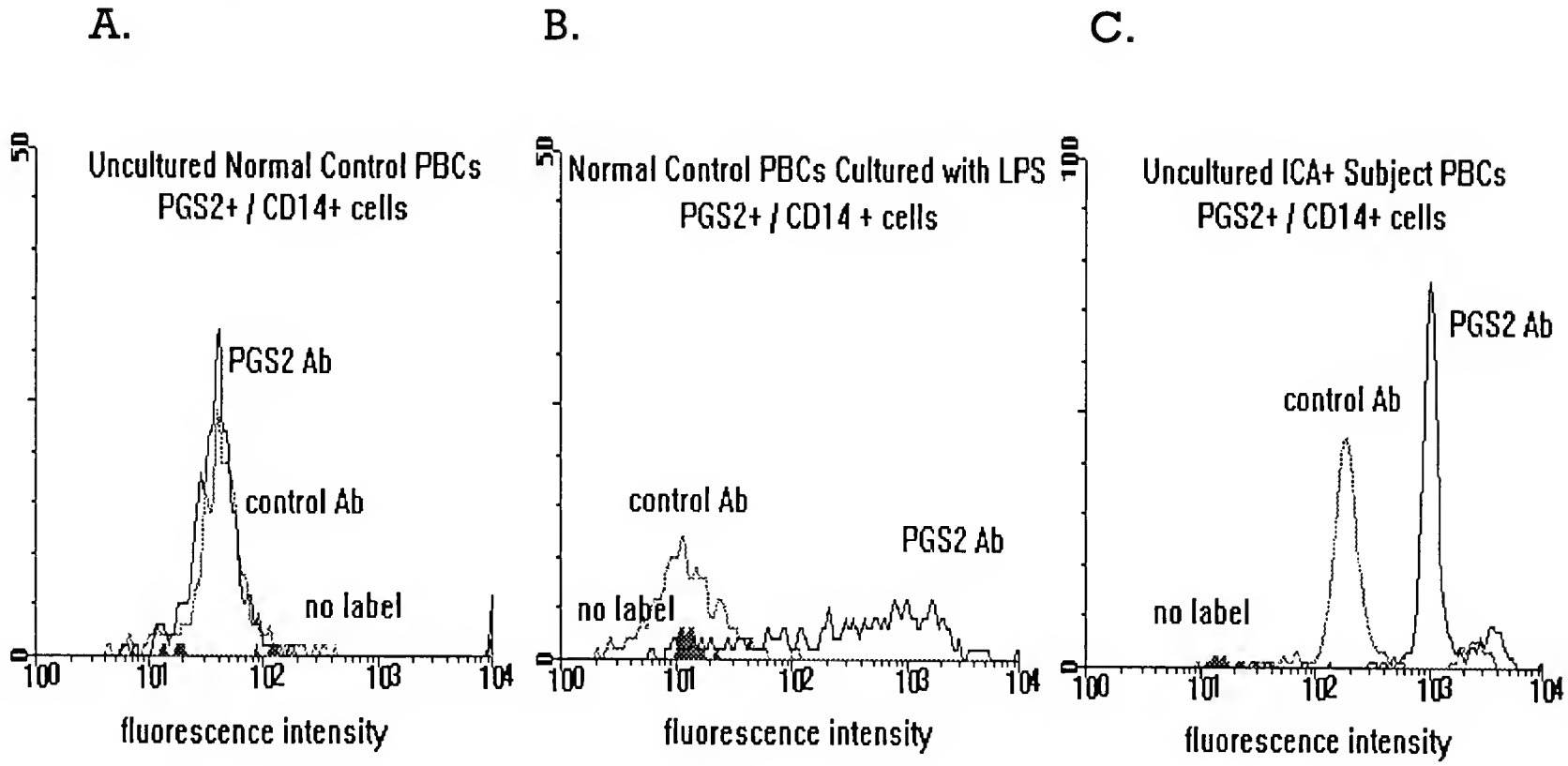
CHAPTER 3
RESULTS

Identification of Aberrant PGS2 Expression in Humans with Autoimmune Disease and Individuals at Risk for Autoimmune Diabetes

FACS analysis of intracellular immunofluorescent staining with monoclonal and polyclonal antibodies specific for PGS2 protein showed both an increase in mean fluorescent intensity (a measure of protein/cell level) and percentage of cells expressing PGS2 in the CD14+ monocytes in autoimmune PBMC sample as compared to those of normal healthy controls (Figure 7).

The increase in percentage of cells positive for PGS2 expression was found to be significant by one-way ANOVA and modified Student t test for sample populations with unequal variances ($p=0.0003$ ANOVA; post t test $p<0.0001$; n controls= 25, n relatives= 5,n total subjects=60;Figure 8). These findings were confirmed by in situ immunohistochemical analysis that showed an increased number of CD14+/PGS2+ cells in autoimmune adherent cell cultures over that in normal control cultures(Figures 9a and 9b). These data indicate the previously detected increase in PGS2 mRNA leads to a quantifiable difference in protein expression in autoimmune cells. Analysis of mRNA from the ex vivo PBMC cells did not yield consistent results due to the low percentage of monocytes in most samples (i.e., visible actin control bands in 5/15 tested). When mRNA was qualitatively detectable, it followed the same pattern as the previous work with adherence isolated monocytes.

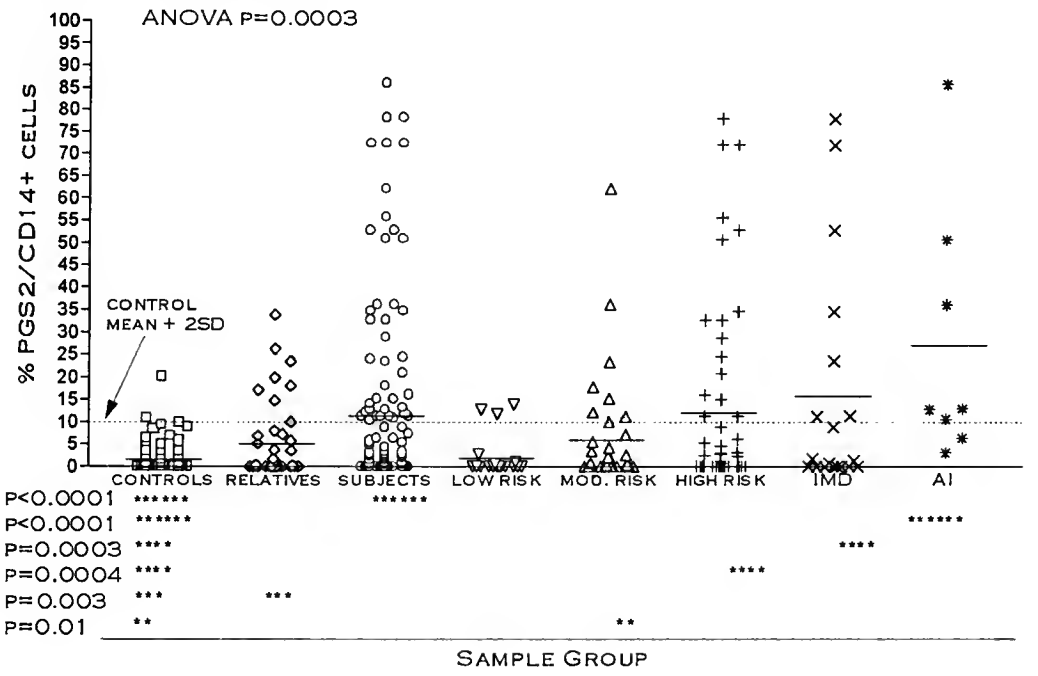
Figure 7. FACS Analysis of Human Peripheral Monocytes for Intracellular PGS2 Protein.



Histograms representative of over 200 similar runs. Shadows peak represents background binding of control non-specific antibody; black line peak represents fluorescence seen with specific anti-PGS2 antibody. A) freshly isolated PBMC of a normal control; B) same control PBMC after 24hr culture with LPS as activation stimulus; C) freshly isolated PBMC of an high risk pre-diabetic ICA+ subject (SQ).

A sample diabetic PBMC gave a visible cDNA product from PGS2 specific primers in the absence of any added monocyte activation factors; whereas, 2 control and 2 relative samples did not.

Figure 8. Percentage of Cells Expressing PGS2 ex vivo is Significantly Higher in Subjects as Compared to Controls. Data from over 200 blood samples taken from test group(60 individuals), relatives (5 individuals), and controls (25 individuals). % PGS2/CD14+ values for relatives, all subjects group, moderate and high risk subjects, diabetics and AI subgroups were found significantly different from the normal control group by one way ANOVA (p=0.0003) and by pairwise posttest for populations with unequal variance; p values as shown below graph. Solid bars indicate mean of group values; dotted line represents control group mean plus 2 standard deviations; level used to define positivity for PGS2 expression.

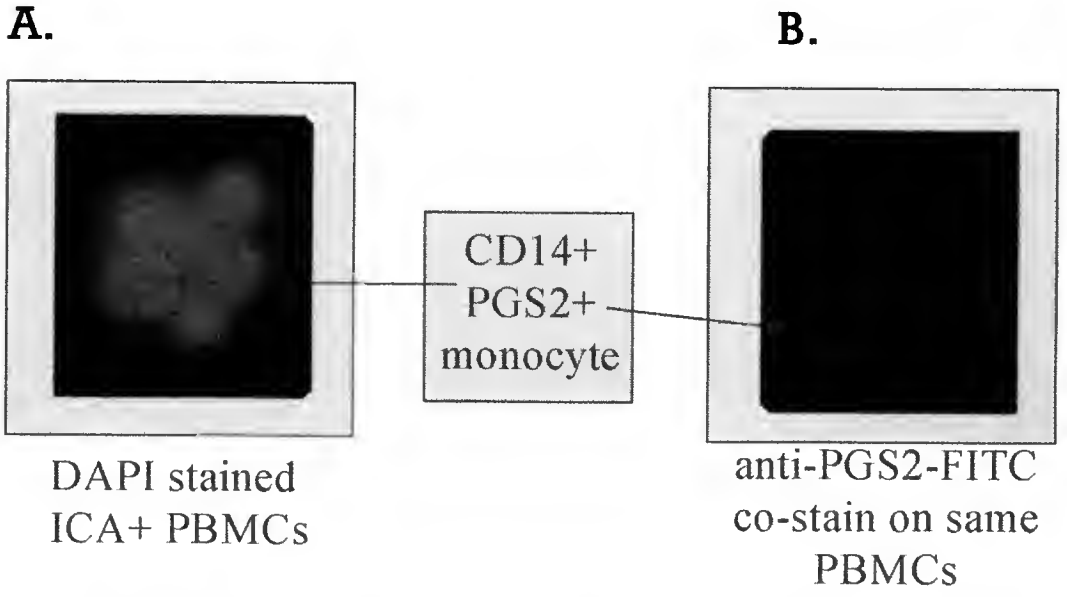


	CONTROLS	RELATIVES	ALL SUBJECTS	LOW RISK	MOD. RISK	HIGH RISK	IMD	AI
SAMPLINGS	54	32	125	18	30	50	19	8
INDIVIDUALS	25	5	60	18	29	15	6	6
# POSITIVE	1	3	23	3	7	12	3	5
% POSITIVE	4%	60%	37%	17%	24%	80%	50%	83%

Autoimmune CD14+ Monocytes Express PGS2 without Co-Expression of Other Activation Markers

Induction of PGS2 expression is considered a marker for macrophage development, with expression occurring later in development (Sweet and Hume, 1996). By co-labeling cells for surface markers and intracellular PGS2,

Figure 9 a and b. Fluorescent Photomicroscopic Visualization of Co-labeling of Freshly Isolated AI PBMC with anti-PGS2-FITC, anti-CD14-PE, and DAPI. Colocalization of all stains on the same cells seen and indicate the intracellular location of the PGS2 protein detected to be surrounding the nuclear contained chromatin stained with DAPI(panel B and A). In contrast, the surface CD14 label is peripheral to these staining.



FACS analysis was used to identify the cell populations expressing the intracellular PGS2, and help define their activation state (Figure 10).

Samples from a total of 42 subjects (ICA+ moderate risk, high risk, diabetic, and autoimmune subject subgroups) and 23 control individuals (CTL) were analyzed. The proportion of CD14+ monocytes expressing intracellular PGS2 was significantly higher in ICA+ subjects as compared to the control group (subjects mean13.4% ±2.9, n=42;58 samplings, controls mean 2.7% ±.65, n=23;45 samplings; p=0.0006). The mean percentage of monocytes (ICA+ vs CTL) expressing DR (21.1 ± 4.5 vs 18.8± 5.6), CD69 (23.3±4.6 vs 28.3 ± 7.5), intracellular TNFα (3.4 ± 0.79 vs 3.8± 1.9), intracellular IL10(1.5± 1.3 vs 1.6 ± 0.61) and CD105 (5.9±1.9 vs 3.3±2.1) was not significantly different in ICA+/PGS2+ subject cells relative to unstimulated ICA-/PGS2- control cells (ICA+ n=22 vs Ctl n=19).

Figure 10. FACS Activation Marker Analysis and Comparison to PGS2 Expression.

sample treatment	PGS2	TNFα	IL10	DR	CD69	CD105
control subjects no stimulus	3.0 ± 0.69; n=42	3.8 ± 1.9; n=19	1.6 ± 0.61; n=11	18.8 ± 5.6; n=12	28.3 ± 7.5; n=17	3.3 ± 2.1; n=8
spontaneous positives*	33.6 ± 9.4; n=6	18 ± 3; n=2	nd	45.9 ± 5.1; n=2	32.6 ± 7.6; n=2	11.3 ± 3.8; n=2
control 10ug/ml LPS stimulus	56.4 ± 9.9; n=4	0 ± 0; n=3	0 ± 0; n=2	4.5; n=1	8.9; n=1	19.2; n=1
control 10ug/ml PHA stimulus	21.6 ± 7.1; n=8	nd	nd	nd	35.7 ± 24.2; n=2	nd
AI subjects no stimulus	15.9 ± 3.3; n=49	3.4 ± 0.79; n=22	1.5 ± 1.3; n=10	21.1 ± 4.5; n=19	23.3 ± 4.6; n=22	5.9 ± 1.9; n=9

Values represent mean, ± SEM; total sample number for FACS determined percentages of CD14+ cells that were also positive for the marker indicated. No stimulus samples are freshly isolated, uncultured PBMC whereas the stimulated samples were cultured for 16-24hrs in medium supplemented with the activation factor indicated. *Spontaneous positive control and relative samples were unmanipulated PBMC from control individuals that had values for percent of CD14+/PGS2+ cells greater than 2SD above the mean for unstimulated controls. nd=not done.

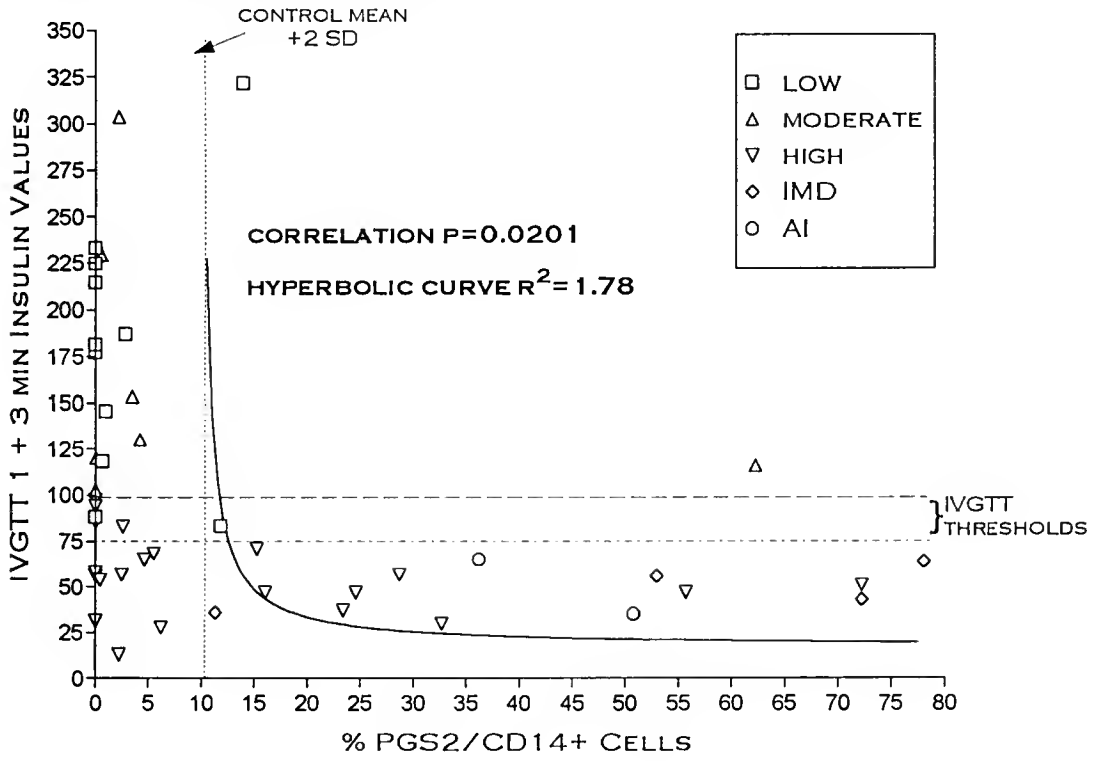
Six out of seventy-four samplings of thirty individuals in the control and relative groups were found to have a spontaneous expression of PGS2 that was greater than 2 standard deviations (SD) above the mean of all control samples. Three of these samples and cultures of controls cells activated with PHA, LPS, and TNFα, were tested for monocyte activation markers. The PGS2 expression of these cells was accompanied by elevated expression of CD69, CD105, DR, intracellular IL10 and intracellular TNFα (Figure 10). In contrast, the spontaneous expression of PGS2 seen in subjects was not accompanied by elevation of any other marker expression above the levels seen in control cell cultures (Figure 10, last row). The reduction of intracellular TNFα and IL10 seen in some of the activated controls maybe a result of activation induced secretion. The lack of activation markers other than PGS2 in the unstimulated controls and subject samples suggested that monocytes are not activated by the isolation processing and the PGS2 levels seen in these samples indicate some other form of expression alteration inherent to the subject cells. These data suggest that the increase in PGE2 expression is occurring out of sequence in the monocyte activation cascade; possibly altering the further activation of the monocyte.

PGS2 Expression Correlates with Clinical and Genetic Markers of IMD High Risk in the Pre-diabetic Population Tested

The 1 + 3 minute insulin levels from IVGTT clinical data reflect the insulin responsiveness of the pre-diabetic individual tested. Values less than 100 for adults and children over 11 years of age and 75 for children under 11

years of age are used as criteria for evaluating risk for diabetes; as are the expression of certain autoantibodies, and HLA genetic subgroups (DR and DQβ). A comparison of PGS2 expression levels with these clinical and genetic markers for IMD risk in a pre-diabetic population shows a marked segregation for the trait with high risk factors for progression to diabetes (Figure11). PGS2 expression is predominant in high risk ICA+ individuals, especially those carrying the HLA alleles DR 04 or 01 and DQβ 0302, 0201, or 05. The expression of PGS2 inversely correlates with IVGTT 1+3min insulin levels (p=0.0201; correlation of highest PGS2 values of 46 subjects, hyperbolic curve regression analysis for best fit; r squared =1.78).

Figure 11. Subpopulation of Pre-diabetic Subjects at High Risk for progression IDDM are Found to be Expressing Uninduced PGS2 and High Levels of PGE2 Production. Low risk (NH group ICA-), moderate risk (NH group, ICA+), high risk (SQ group and ICA+, low IVGTT NH group members) subjects, diabetics (IMD), and individuals with other autoimmune diseases were analyzed as to their IVGTT 1 + 3 minute insulin levels correlation with PGS2 expression. The correlation was found to be significant (p=0.0201, n= 46 total subjects) and to best fit a hyperbolic curve (r squared value=1.78).



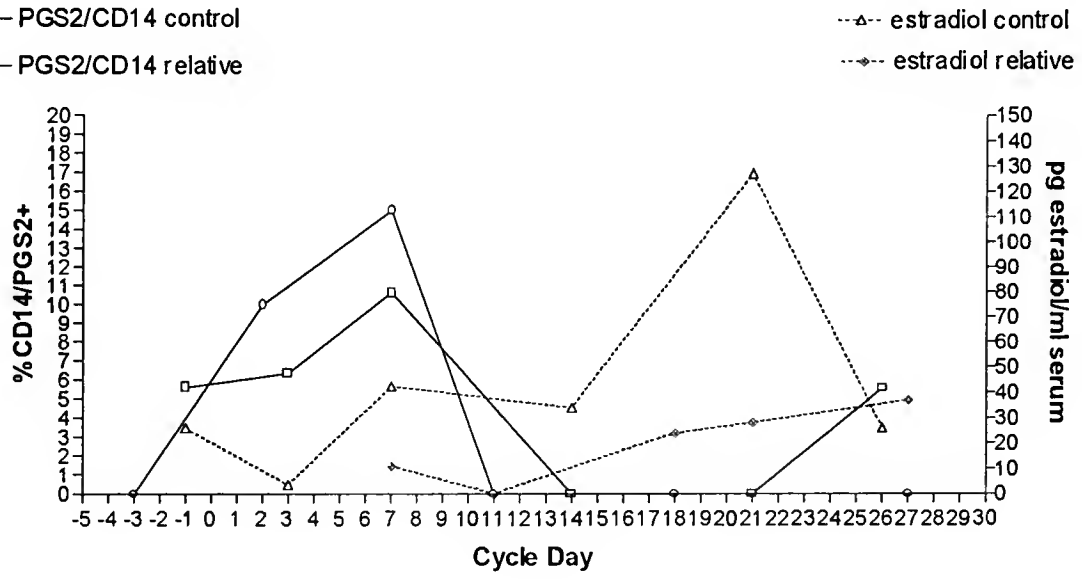
Age and Gender Affects the Expression of PGS2 by Autoimmune PBMC

The test subject group had a sampling bias of females (41 individuals) to males (26 individuals). The age range was not random among the sexes, with a bias toward older females (>30 years of age) and younger males (< 20 years of age). These sex and age bias did not show any significant differences in PGS2 expression when tested by Student t test or ANOVA (gender and age subgroups; 108 samplings of subjects). Control females had PGS2 expression significantly lower than that seen in subject females (n controls = 29, mean 2.35%, SD=4.5; n subjects=62, mean 11.56%, SD=21.5; p= 0.012, Student t test). Based on the finding that the highest expression of macrophage PGS2 is found in the estrus phase of the female NOD mouse, PBMC from human females were tested to determine if a similar variation in PGS2 expression was linked to their menstrual cycle. Analysis of one control female and one control relative was done over a period of 30 days, testing PBMC for PGS2 expression and serum for estradiol levels (Figure 12).

The observation drawn from this and collaborative interviews with female subjects and other controls (concerning estrogen and progesterone therapies and cycle history) is that PGS2 expression appears to vary with the menstrual cycle. These data suggest an increase in PGS2 expression occurs prior to the luteal phase of the cycle. More data on a larger sample population is needed to accurately determine the exact peak of PGS2 expression.
In vivo esterdiol or progesterone treatment of PBMC from both males and females in control and test subject groups gave variable PGS2 expression results by FACS, suggesting that there is individual variation in

responsiveness to the hormones. Progesterone concentration may play a role in this responsiveness, with sensitivity being dependent on estrogen induced progesterone receptor expression or other unknown factors.

Figure 12. Analysis of Monocyte PGS2 Expression During the Menstrual Cycle. Two control female volunteers, one normal healthy control and one nonautoimmune relative control, were monitored over a 30 day period for PGS2 Expression in PBMC and esterdiol level in serum. Cycle day was recorded from the individual's report of the first day of their period designated as day zero.



Insensitivity of PGS2 Expression to Suppression by IL10

ELISA analysis of IL10 production by PBMC in culture suggested that subject cells produce the immunosuppressive cytokine at levels equal to or greater than normal controls and nonautoimmune relatives (n controls = 37; mean 92.1pg/million cells, SD=94.1; n subjects = 43; mean 98.5pg/million cells , SD=120.2).

In vitro studies of PBMC cultured in the presence of 500ng/ml IL10 alone or with LPS activation stimulus showed that PGS2 expression in 6 of 12 subject samples assayed were insensitive to IL10 suppression. Of 12 subjects and 8 controls samples cultured, the controls on average decreased their

PGS2 expression down to 45% of the baseline levels (SD=12%); whereas, subject samples on average increased their PGS2 expression to 2.64 times the level seen in their baseline cultures (SD=1.7). When stimulated by LPS, the differential PGS2 expression maintained the same insensitivity pattern; with controls decreasing their expression (mean 0.52 fold, SD=0.26) and subjects remaining at the elevated levels induced in them by LPS (92% of LPS stimulated level, SD=68%). The high variability in the data suggests that factors other than IL10 insensitivity are involved in PGS2 expression.

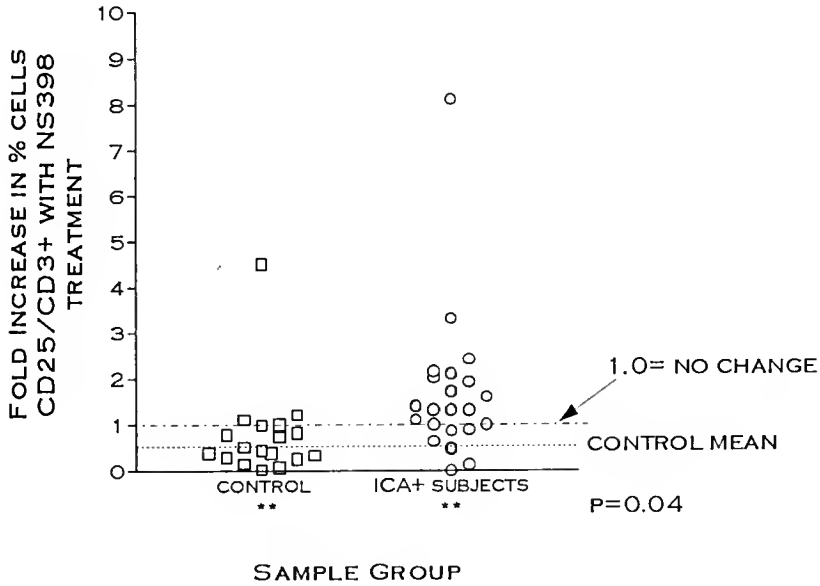
PGE2 Inhibition of CD25 Expression on Activated Subject T cells

PBMC from controls, nonautoimmune relatives, and subjects were tested in vitro for the effect of the PGS2 inhibition on IL2 signal transduction in T cell AICD and proliferation. These cultures were stimulated with the T cell mitogen, PHA, at levels that would induce both proliferation and AICD (5-10ug/ml). The samples were set with or without the specific PGS2 inhibitor, NS398 (5μM) and then assayed by FACS and ELISA for T cell activation, proliferation and cell death. Tritiated thymidine uptake was lower overall in the subject samples (n=7, mean uptake 7021cpm) than in controls (n=3, mean uptake 10618 cpm) but no large changes were seen with NS398 treatment (subjects 1.2 fold increase, controls 0.95 fold change). ELISA for PGE2 indicated that the PGE2 was present in unactivated and activated subjects and activated controls, but were lowered or totally inhibited in NS398 treated cultures. IL4 levels were undetectable or at the limit of detection by ELISA in all samples(1/4 controls;0/8 subjects). IFNγ was not detected in most cultures; however, in samples with expression, NS398 treatment caused a increase in IFNγ (5/8 subjects mean increase 42.3 fold; 3/4 controls mean change 0.94 fold). Basal IL2 expression as measured by ELISA was low to undetectable in most of the samples tested; however, in subject samples with IL2 detectable in

PHA activated culture, NS398 treatment caused an increase in its expression (11/16 subjects, mean increase 77 fold). In contrast, control samples had little change in IL2 expression in the presence of NS398 (2/4 controls mean change 1.2 fold).

FACS analysis for IL2 receptor α protein, CD25, showed a significant increase in CD25 expression on subject CD3+ cells with NS398 treatment during PHA stimulation (Figure 13;p=0.04, Student t test). Subject CD3+ cells increased their CD25 expression by an average of 2.0 times (SD=3.1, n=30); whereas, controls decreased their expression to 0.45 fold (SD=0.37, n=10) and relatives decreased theirs to 0.49 fold (SD=1.4, n=8) compared to expression with PHA stimulation alone. The increase in CD25 expression was especially strong in ICA+ SQ group individuals with the HLA alleles of DR4, DQβ0302 (p=0.01).

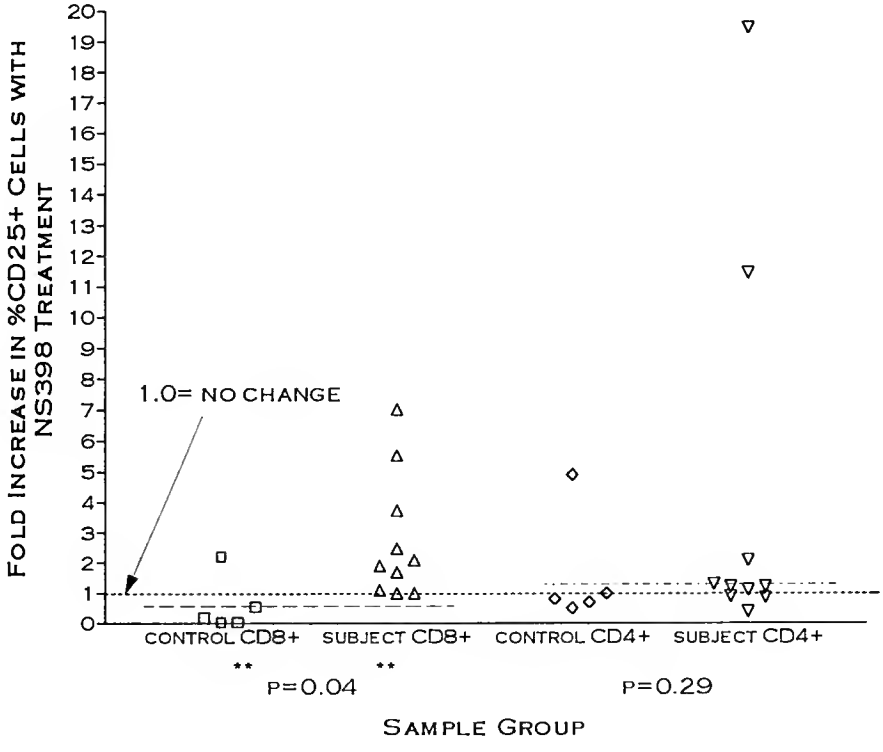
Figure 13. CD25 Expression Induction on CD3+ T Cells with PHA Stimulation and Treatment with NS398. Cells were cultured in the presence of PHA and with or without NS398. The Fold Increase reported in this graph was calculated as the % of cells with CD25/CD3+ phenotype in NS398 and PHA relative to culture with PHA alone. 'Controls' in this graph represent normal controls and nonautoimmune relative. 'ICA+ Subjects' represents ICA+ diabetics, autoimmune subjects, moderate and high risk individuals.



CD8+ T cell Subpopulation Bias in CD25 Expression in Autoimmune PBMC

FACS analysis was used to detect CD25 expression on T cell subtypes in PBMC mixed cell cultures grown under conditions identical to those used for the CD3+ analysis. These data show that the significant increase in CD25/CD3+ expression is due to an increase in CD25 expression on CD8+ T cells (p=0.01,Figure 14). In contrast, though the CD4 cells increase as well, this increase was not significantly different from the changes seen in control and relative samples under the same conditions(p=0.29, Figure 14).

Figure 14. CD25 Expression Increase with NS398 Treatment with PHA Stimulation on CD8+ and CD4+ T cells. Twenty-four hour cultures of PBMC with 5ug/ml PHA and with or without 5uM NS398. Data presented as fold increase with the addition of NS398 to the PHA stimulated cultures as compared with PHA alone. Dotted lines represent the mean of the control samples tested. The p values listed below the graph are from Student t test analysis of the control and subject sample pairs (CD8+ and CD4+). A significant difference was seen in the increased %CD25 expression on CD8+ in the test group relative to controls (p=0.04). CD4+ cell expression of CD25 was also increased with NS398 present; however, this was not significantly different from controls.



PGE2 Mediated CD25 Suppression Contributes to its Block of AICD

To test whether the influence of PGE2 on CD25 was linked to a suppression of AICD, PBMC cultures were set up as before but maintained 24hr or 5 days in the presence of 5ug/ml PHA with or without 5μM NS398 treatment. After ficoll removal of dead cells, the cultures were transferred to cell dishes coated with anti-*FAS* antibody, a known apoptosis inducer of activated T cells. Wells without antibody were seeded in parallel and after 24hr incubation, the cells were again analyzed for CD25 expression and for induction of cell death. Plate bound anti-*FAS* antibody substituted for FAS-L crosslinking of *FAS* receptors; thereby, providing a standardized AICD stimulus. The anti-*FAS* treatment allowed for greater cell death of CD25+ cells when NS398 was present in subject cell cultures than when cultured with PHA alone prior to the exposure to antibody (Figure 15; control % PI<2n CD25+ cells fold index mean=0.94, SD 0.16; subject mean=2.4, SD 2.76). *FAS* expression on subject cells was not found to be significantly different from that of control cells, regardless if grown under unstimulated or PHA stimulated conditions. Measurement of *FASL* expression was attempted but the results were ambiguous due to poor antibody binding. CD25 cells in many of the subject samples were also high in *FAS* expression, but unlike CD25+ cells in controls, were not susceptible to AICD induction by PHA or anti-*FAS* without NS398 present.

When galardin treatment used in place of NS398, increased *FAS* and CD25 expression on both controls and subjects but not enhancement anti-*FAS* mediated AICD induction in subjects. Metalloproteinase activity was not measured in these cultures.

Figure 15. Fold Increase in AICD Induction for CD25+ Cells After PHA Stimulation and with NS398 Treatment.

sample	medium alone	PHA	PHA+NS398	Fold Increase(NSPHA/PHA)
controls				*%CD25+ cells with <2n PI intercalation*
CF28	1.38	1.84	1.19	0.65
CM13	34.47	35.32	34.89	0.99
CRM26	24.83	43.21	9.09	0.21
CRM27**	37.98	40.38	51	1.26
CRF1; 5day culture	8.05	22.18	31.66	1.43
CM17;5day culture	8.53	22.18	33.16	1.50
CRM4;5day culture	34.47	35.32	34.89	0.99
mean	21.39	28.63	27.98	1.00
standard deviation	15.13	14.38	17.03	0.46
subjects				
NHM5**; 5day culture	18.66	3.06	24.45	7.99
NHF54;5day culture	18.81	19.09	28.3	1.48
NHM3**	37.98	40.38	51	1.26
SQF5	46.54	16.09	31	1.93
SQM30	52.73	56.55	50.69	0.90
DF60**	0.86	1.08	0.97	0.90
mean	29.26	22.71	31.07	2.41
standard deviation	19.77	21.76	18.65	2.76

**positive for PGS2 expression(greater than control mean +2SD)

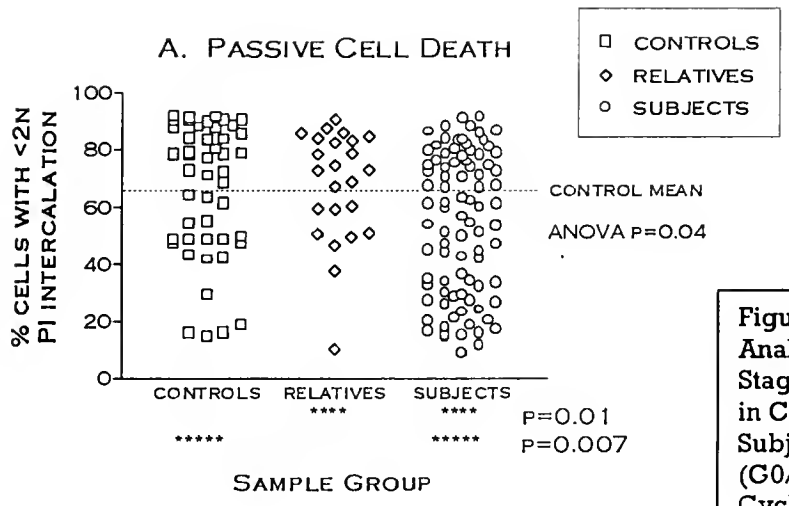
Comparison of % CD25+ cells undergoing AICD induced by anti-*FAS* antibodies. Data represents Fold increase in % of PHA stimulated cells with PI intercalation less than 2n and CD25+ in the presence and absence of NS398 when induced to AICD by plating of antibody coated cell plates. Unless otherwise indicated, cultures were incubated 24hr at 37C/5%CO2 prior to 24hr exposure to anti-FAS antibody coated plates.

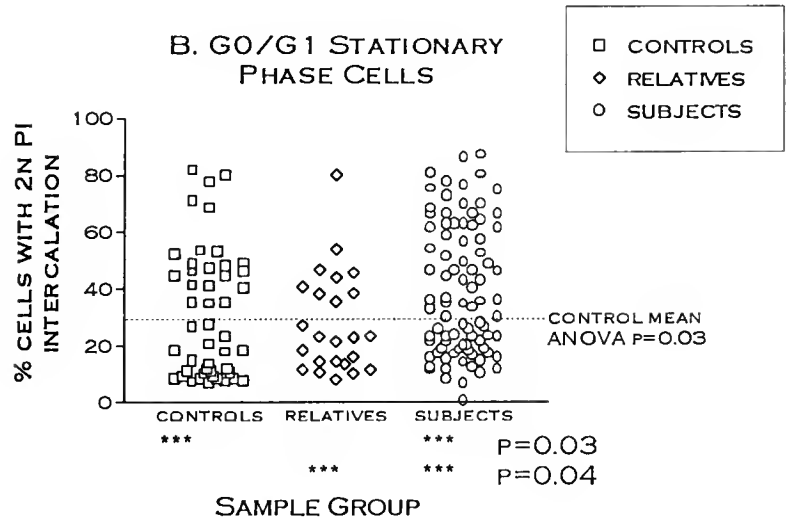
Decreased Spontaneous Cell Death and Increase of Cells in G0/G1 and in Cycle in Autoimmune PBMC

A significant decrease in spontaneous PCD was noted in the subject group compared with controls or with relatives by FACS analysis of propidium iodide intercalation(Figure 16, panel A: ANOVA p=0.04;sample n of controls=47, mean=66% SD=23; n relatives= 24, mean 68% SD=19; n subjects= 87, mean=54% SD=25). A concurrent significant increase in both the cell populations remaining in G0/G1(Figure 16, panel B; 2n chromatin; ANOVA p=0.03) and in cells entering cycle (Figure 16, panel C; PI intercalation >2n level;ANOVA p=0.02). These data were collaborated by TUNEL FACS analysis and morphological staining of cells, suggesting that the decrease in PCD seen in these assays is due to a decrease in apoptotic death.

Ceramide Levels are Elevated in Autoimmune PBMC

NOD mice were found to have increased levels of the AICD intermediate, ceramide, as compared to BALB/c and B6 control strains. It is possible that the changes in PGE2 expression could be a part of a larger problem with lipid metabolism and AICD induction. To examine this possibility in humans, lipid analysis was done on the subject and control PBMC in parallel to the PGS2 expression FACS analysis. Lipid extracts from fresh PBMC of controls and subjects were analyzed by HPTLC. Lipid spots that comigrated with ceramide standards were found to be increased in subjects as compared to controls (n controls=7, mean 1.034μg/million cells, SD=1.27; n





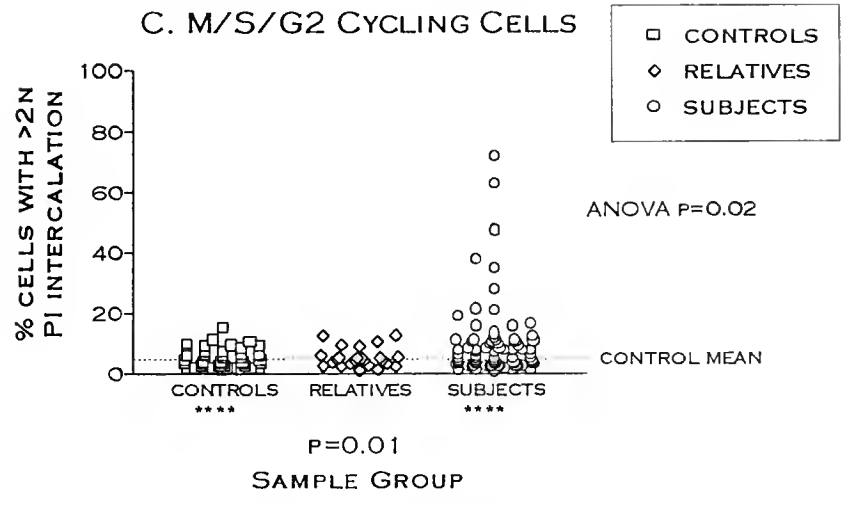
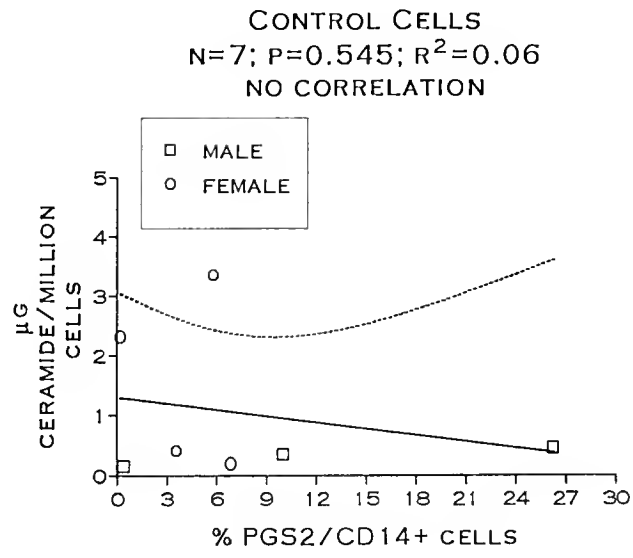


Figure 16. Propidium Iodide Analysis of Cell Cycle Stages. A. Passive Cell Death in Controls, Relatives, and Subjects. B. Stationary Phase (G0/G1) Cells. C. Cells in Cycle.(M, S, & G2).

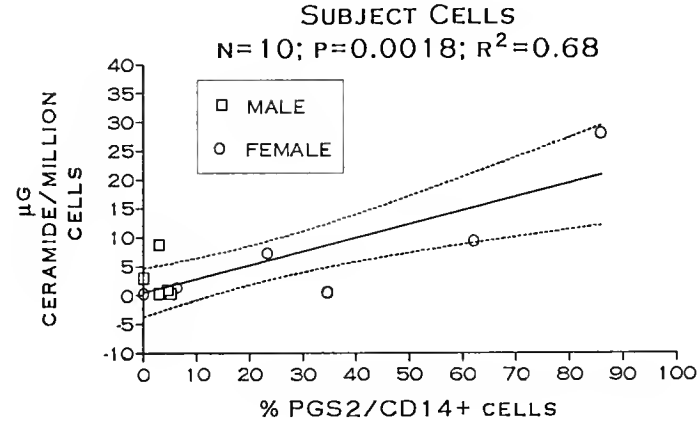
subjects=11, mean 5.391μg/million cells, SD= 8.29). The change in ceramide levels was different between genders, with females being higher than males in both groups (control females mean 1.571 μg/ml; control males mean 0.317; subject females mean 7.755; subject males mean 2.554). Linear regression analysis depicted in Figure 17 shows this increase was positively correlated with the increase in PGS2 expression observed in these subjects.

Figure 17. Correlation of Ceramide Levels with PGS2 Expression. A. in Controls. and B. in Subjects. Data represents average of duplicate samples run on the same HPTLC plate. Linear regression correlation analysis of 10 subject and 7 control samples is depicted. Dotted curves represent 95% confidence intervals.

A.



B.



CHAPTER 4
DISCUSSION

The aberrant PGS2 mRNA expression and associated PGE2 overproduction was recently described by Xie (1997) in the NOD mouse and by Clare-Salzler(1995) in human peripheral blood monocytes of individuals at risk of IMD, those with overt IMD, and with other autoimmune diseases including rheumatoid arthritis, vitiligo, ulcerative colitis, Addison's Disease, Graves' Disease, Hashimoto's thyroiditis, and SLE. This study gives a quantitative definition to the PGS2 expression defect in humans and provides insight into possible mechanisms of its role in immunopathogensis.

Intracellular FACS analysis of freshly isolated human PBMCs allowed for detection of ex vivo quantitation of PGS2 expression without the background activation caused by adherence purification of monocytes or the transient increase in expression that accompanies in vitro culturing. Aberrant PGS2 protein expression in unactivated peripheral blood monocytes was found in 37% of the test subjects as compared with 4% of normal healthy controls ($p<0.0001$). The presence of the enzyme without indicators of monocyte activation gives support to the idea that the monocyte/macrophage population has been altered in autoimmune individuals, yielding a defect in APC function which affects T cell activation. The presence of this aberrant PGS2 expression correlated inversely with insulin responsiveness as measured by IVGTT ($p=0.0201$), indicating a link between the defect and

immunopathogensis of IMD. This link presents the opportunity for use of PGS2 expression as a barometer for disease activity; both as a diagnostic indicator of risk for IMD and a potential target for therapeutic intervention. Nonsteroidal anti-inflammatory drugs (NSAID) such as the PGS2 specific inhibitor NS398 and related compounds, may constitute a new pharmacological avenue for the treatment and prevention of autoimmune disease.

In many autoimmune diseases there is a gender bias and/or an age of onset effect. IMD does not have a defined gender bias but does have an age of onset prevalence that coincidences with puberty (Winter et al., 1993). The sample population tested in this study had a bias in the population toward sexually mature females and pre-pubescent males. This may represent a sampling bias, not the incidence level in the total at-risk population. Adult control females were found to have cyclic variations in their PGS2 expression that coincided with their menstrual cycle. Estrogen and progesterone therapies, such as birth control use and estrogen replacement therapy, are postulated to affect PGS2 expression. These influences on PGS2 expression contribute to the sample variance seen in the study population. It is interesting to note that even with this potential signal interference in the data, female subjects were found to have a significantly higher level of PGS2 expression than female controls ($p=0.01$). Though no gender or age bias for IMD could be defined from this study, the observations suggest a more in-depth study is needed into the effects of sexual maturation and the use of hormone-based therapies in the risk assessment for IMD and other autoimmune diseases.

The underlying cause of the PGS2 expression defect is still unknown. The immunosuppressive cytokine IL10 has been the object of recent transgenic and congenic analysis in the NOD mouse (Lee et al., 1996). The

PGS2 expression of some test group samples in vitro showed insensitivity to interleukin10 suppression, a normal regulatory control of PGS2 expression. Further investigation is needed to determine if such a regulatory dysfunction is an underlying mechanism for the defect in vivo.

When PGS2 expression was inhibited in vitro, test subject T cells increased IL2 receptor expression (CD25) and their susceptibility to AICD ($p=0.04$). This effect is especially prevalent in cells from subjects with the IMD susceptibility major histocompatibility locus alleles DR4 and DQβ0302 ($p<0.001$). This prevalence suggests a linked effect between PGE2 interference with IL2 signaling and the high risk HLA allele on T cell activation. Moreover, the greatest change in CD25 expression attributable to PGS2 expression was seen in the subject CD8+ T cells ($p=0.01$). CD8+ T cells are thought to be the primary subpopulation involved in suppression regulation of T cell functions, including T helper cell differentiation to Th1 or Th2 cells (Fukuse et al., 1992; Eimasry et al., 1986; Eimasry et al., 1987; Balashov et al., 1995; Goetzl et al., 1995). A selective influence on the activation and elimination potential of CD8+ regulatory T cells would have dramatic effects on the regulatory control of peripheral tolerance, T helper cell functional bias (Th1 verses Th2), as well as appropriate conclusion of immune responses and memory cell development. Interference with these types of immune regulation could promote autoimmunity and immunopathogensis.

Passive cell death, defined as spontaneous death of cells ex vivo, was significantly lower in test subject cells than nonautoimmune relatives and normal controls($p=0.04$). Concurrently, the percentage of subject cells remaining in G0/G1 and entering cycle were significantly higher($p=0.03$ and 0.02, respectively). Preliminary data suggests that subject cells in culture

were also resistant to AICD induced by anti-*FAS* antibody. These data suggest that autoimmune PBMC are unusually resistant to cell killing. The precedence for lack of cell death leading to autoimmune disease has been shown in the *lpr* and *gld* mouse models and more recently in humans with SLE and SLE-like lymphoproliferative disease (Howie et al., 1994; Sneller et al., 1992; Emlen et al., 1994). Resistance to AICD in vitro was reversible in this study by inhibition of PGS2 expression. PGE2 effects on IL2, CD25, metalloproteinase cleavage of surface ligands, and receptors all can contribute to its effects on AICD signaling. However, surface signal effects alone did not account for all of the PGE2 effects on AICD. Galardin treatment prevented loss of surface molecules including FAS and CD25 from subject cells, but did not promote greater susceptibility to AICD induction. Though Galardin is a broad spectrum inhibitor of metalloproteinase, its anti-proteolytic effects may not affect the molecules involved in the resistance to AICD seen in these cells. Another possible mechanism for the effectiveness of NS398 inhibition of PGS2 expression in promoting AICD susceptibility is that PGE2 may be affecting AICD signal transduction at a point beyond the initial surface receptor-ligand interaction. The accumulation of ceramide, a second messenger in AICD signal transduction, directly correlated with the increase in PGS2 expression found in the test subject individuals($p=0.002$). Ceramide functions in promotion of kinases and dephosphorylases important for AICD and in arresting cell cycle progression (Hannun and Obeid, 1995). Recent data show that PGS2 expression is promoted by ceramide; whereas, the action of ceramide as a nuclear signal transduction molecule are somehow blocked by PGE2 (Ballou et al., 1992; Hannun, 1997). This interplay of lipid metabolytes of AA represents another level of signal transduction/cell activation regulation that is just now being uncovered. Ceramide has been

implicated in control of *ICE*, MAP kinases, and *RAS/RAF* related proteins (Hunnan, 1997). PGE2, through its actions on cAMP, also affects the same metabolic pathways. The complex interplay and balance of these lipid components may be similar to the *BCL2-BAX/BCL-x* protein level balances needed to maintain a cell in a viable, functional state. The mechanism of how PGE2 is promoting resistance to cell death and; thereby, setting the stage for development of autoimmune dysfunction lies in its role in signal transduction. Further studies into the lipid metabolism of cells is needed to elucidate this role.

This study confirms the identification of aberrant monocyte PGS2 expression as a risk factor for IMD and presents evidence for its role as a component of the immunopathogensis of multiple autoimmune diseases.

REFERENCES CITED

Ahmed, N. N., H. L. Grimes, A. Bellacosa, T. O. Chan, and P. N. Tsichilis. 1997. " Transduction of Interleukin-2 Antiapoptotic and Proliferative Signals via Akt Protein Kinase." *Proceedings of the National Academy of Sciences USA.* 94: 3627 3632.

Alderson, M. R., R. J. Armitage, E. Maraskovsky, T. W. Tough, E. Roux, K. Schooley, T. Ramsdell, and D. H. Lynch. 1993. "Fas Transduces Activation Signals in Normal Human T Lymphocytes." *Journal of Experimental Medicine.* 178: 2231-2235.

Anastasia, E. D., F. Paliogianni, J. P. Balow, H. Yamada, and D. T. Boumpas. 1992. "Prostaglandin E2 and Other Cyclic AMP-Elevating Agents Modulate IL-2 and IL-2Ra Gene Expression At Multiple Levels." *Journal of Immunology.* 148(9): 2845-2852.

Anastassiou, E. D., F. Paliogianni, J. P. Balow, H. Yamada, and D. T. Boumpas. 1992. "Prostaglandin E2 and Other Cyclic AMP-Elevating Agents Modulate IL-2 and IL-2Rα Gene Expression at Multiple Levels." *Journal of Immunology.* 148: 2845-2952.

Antonaci, S., C. Tortorella, A. Poignano, A. Ottolenghi, E. Jirillo, and L. Bonomo. 1991. "Modulating Effects on CD25 and CD71 Antigen Expression by Lectin Stimulated T Lymphocytes in the Elderly." *Immunopharmacology and Immunotoxicology.* 13(1&2): 87-100.

Baixeras, E., L. Bosca, C.Staiber. A. Gonzalez, A.C. Carrera, J.A. Gonzalo, and C. Martinez-A. 1994. "From Apoptosis to Autoimmunity: Insights from the Signaling Pathways Leading to Proliferation or to Programmed Death. p.53-92. in "Apoptosis in Immunity"; dedicated volume of *Immunological Reviews*; G. Moller; ed. 142: December.

Balashov, K. E., S. J. Khoury, D. A. Hafler, and H. L. Weiner. 1995. "Inhibition of T cell Responses by Activated Human CD8+ T Cells is Mediated by Interferon-γ and is Defective in Chronic Progressive Multiple Sclerosis." *Journal of Clinical Investigation.* 95: 2711-2719.

Ballou, L. R., C. P. Chao, M. A. Holness, S. C. Barker, and R. Raghow. 1992. "Interleukin-1-mediated PGE2 Production and Sphingomyelin Metabolism." *Journal of Biological Chemistry.* 267(28): 20044-20050.

Berger, S., H. Ballo, and H. J. Stutte. 1996. "Immune Complex-induced Interleukin-6, Interleukin-10, and Prostaglandin Secretion by Human Monocytes: a Network of Pro- and Anti-inflammatory Cytokines Dependent on the Antigen: Antibody Ratio." *European Journal of Immunology.* 26: 1297-1301.

Bligh, E. G. and W. J. Dyer. 1959. "A Rapid Method of Total Lipid Extraction and Purification." *Canadian Journal of Biochemistry and Physiology.* 37(8): 911-917.

Bose, R., M. Verheij, A. Haimovitz-Friedman, K. Scotto, Z. Fuks, and R. Kolesnick. 1995. "Ceramide Synthase Mediates Daunorubicin-Induced Apoptosis: An Alternative Mechanism for Generating Death Signals." *Cell.* 82: 406-414.

Brunner,T., R.J. Mogil, D. LaFace, N. J. Yoo, A. Mahboubl, F. Echeverri, S. J. Martin, W. R. Force, D. H. Lynch, C. F. Ware, and D.R. Green. 1995. "Cell-autonomous *Fas* (CD95)/Fas-ligand Interaction Mediates Activation-induced Apoptosis in T-cell Hybridomas." *Nature.* 373: 441-448.

Chen, D. and E. V. Rothenberg. 1994. "Interleukin 2 Transcription Factors as Molecular Targets of cAMP Inhibition: Delayed Inhibition Kinetics and Combinatorial Transcription Roles." *Journal of Experimental Medicine.* 179: 931-942.

Chen, M., J. Quintans, Z. Fuks, C. Thompson, D. W. Kufe, and R. R. Weichselbaum. 1995. "Suppression of *Bcl-2* Messenger RNA Production May Mediate Apoptosis after Ionizing Radiation, Tumor Necrosis Factor α, and Ceramide." *Cancer Research.* 55: 991-994.

Cheng, J., C. Liu, W. J. Koopman, and J. D. Mountz. 1995. "Characterization of Human Fas Gene: Exon/Intron Organization and Promoter Region." *Journal of Immunology.* 154: 1239-1245.

Chervonsky, A. V., Y. Wang, F. S. Wong, I. Visintin, R. A. Flavell, C. A. Janeway, Jr., and L. A. Matis. 1997. "The Role of *Fas* in Autoimmune Diabetes." *Cell.* 89: 17-24.

Cifone,M. G., R. De Maria, P. Roncaioli, M. R. Rippo, M. Azuma, L. L. Lanier, A. Santoni, and R. Testi. 1993. "Apoptotic Signaling through CD95

(*Fas/Apo-1*) Activates an Acidic Sphingomyelinase." *Journal of Experimental Medicine.* 177: 1547-1552.

Clare-Salzler, M. J. 1994. "Monocyte Dysfunction in Pre-IDDM; Spontaneous Expression of Prostaglandin Synthase-2." manuscript in preparation.

Clare-Salzler, M. J. 1995. "The Role of NOD Macrophages in the Dysregulation of Peripheral Tolerance." manuscript in preparation.

Clare-Salzler, M. J., J.Brooks, A. Chai, K. Van Herle, and C. Anderson. 1992. "Prevention of Diabetes in Nonobese Diabetic Mice by Dendritic Cell Transfer." *Journal Clinical Investigation.* 90: 741-748.

Clare-Salzler, M. J. and Y. Mullen. 1992. "Marked Dendritic Cell-T cell Cluster Formation in the Pancreatic Lymph Node of the Non-Obese Diabetic Mouse." *Immunology.* 76: 478-484.

Clare-Salzler, M. J., J.Brooks, A. Chai, K. Van Herle, and C. Anderson. 1992. "Prevention of Diabetes in Nonobese Diabetic Mice by Dendritic Cell Transfer." *Journal Clinical Investigation.* 90: 741-748.

Cohen, I. R. and D. B. Young. 1992. "Autoimmunity, Microbial Immunity and the Immunological Homunculus." *Immunology Today.* 13: 490.

Cook, S. J. and F. McCormick. 1993. "Inhibition by cAMP of Ras-Dependent Activation of Raf." *Science.* 262: 1069-1072.

Cremion, C., A. Habib, J. Maclouf, P. Pradelles, J. Grassi, and Y. Frobert. 1995. Differential measurement of constitutive (COX-1) and inducible (COX-2) cyclooxygenase expression in human umbilical endothelial cells using specific immunometric enzyme immunoassays. *Biochim Biophys Acta.* 1264: 341-348.

Daftarian, P. M., A. Kumar, M. Kryworuchko, and F. Diaz-Mitoma. 1996. "IL10 Production is Enhanced in Human T Cells by IL-12 and IL-6 and in Monocytes by Tumor Necrosis Factor-α." *Journal of Immunology.* 157: 12-20.

Dai, W. J., G. Kohler, and F. Brombacher. 1997. "Both Innate and Acquired Immunity to *Listeria monocytogenes* Infection are Increased in IL-10-Dificient Mice." *Journal of Immunology.* 158: 2259-2267.

D'Andrea, A., M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin and G. Trinchieri. 1993. "Interleukin 10 (IL-10) Inhibits Human Lymphocyte Interferon γ-Production by Suppressing Natural Killer Cell Stimulatory

Factor/IL-12 Synthesis in Accessory Cells." *Journal of Experimental Medicine.* 178: 1041-1048.

de Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries. 1991. "Interleukin 10 (IL-10) Inhibits Cytokine Synthesis by Human Monocytes." *Journal of Experimental Medicine.* 174: 1209-1219.

DeWitt, D. and W. L. Smith. 1995. "Yes, but Do They Still Get Headaches?" *Cell.* 83: 345-348.

Dhein, J., H. Walczak, C. Baumler, K.-M. Debatin, and P. H. Krammer. 1995. "Autocrine T-cell Suicide Mediated by APO-1/(Fas/CD95)." *Nature.* 373: 438-440.

Ehrhardt, R. O., B. R. Ludviksson, B. Gray, M. Neurath, and W. Strober. 1997. "Induction and Prevention of Colonic Inflammation in IL-2-Deficient Mice." *Journal of Immunology.* 258: 566-573.

Eimasry, M. N., E. J. Fox and R. R. Rich. 1986. "Opposing Immunoregulatory Functions of CD8+ Lymphocytes: A Requirement for Monocytes in Suppressor Cell Induction." *Journal of Immunology.* 137(8): 2468-2477.

Eimasry, M. N., E. J. Fox and R. R. Rich. 1987. "Sequential Effects of Prostaglandins and Interferon-γ on Differentiation of CD8+ Suppressor Cells." *Journal of Immunology.* 139(3): 688-694.

Emlen, W., J. Niebur, and R. Kadera. 1994. "Accelerated In Vitro Apoptosis of Lymphocytes from Patients with Systemic Lupus Erythematosus." *Journal of Immunology.* 152: 3685-3692.

Faustman, D. 1993. "Mechanisms of Autoimmunity in Type I Diabetes." *Journal of Clinical Immunology.* 13(1): 1-7.

Faveeuw, C., M.-C. Gagnerault, and F. Lepault. 1994. "Expression of Homing and Adhesion Molecules in Infiltrated Islets of Langerhans and Salivary Glands of Nonobese Diabetic Mice." *Journal of Immunology.* 152: 5969-5977.

Foegh, M. L. 1988. "Immune Regulation by Eicosanoids." *Transplantation Proceedings.* 20(6): 1158-1161.

Fournel, S., L. Genestier, E. Robinet, M. Flacher, and J.-P. Revillard. 1996. "Human T cells Require IL-2 but not G1/S Transition to Acquire

Susceptibility to *Fas*-Mediated Apoptosis." *Journal of Immunology.* 157: 4309-4315.

Fukuse, S., C. Terajima, A. Koontz, M. Kelley, D. R. Webb, and B. H. Devens. 1992. "Interleukin-2 Stimulates the Development of Anergy via the Activation of Nonspecific Suppressor T Cells." *International Archives of Allergy Immunology.* 99: 411-415.

Futaki, N., S. Takahashi, M. Yokoyama, I. Arai, S. Higuchi, and S. Otomo. 1994. "NS-398, A New Anti-inflammatory Agent, Selectively Inhibits Prostaglandin G/H Synthase/Cyclooxygenase (COX-2) Activity in vitro." *Prostaglandins.* 47: 55-59.

Garchon,H.-J., J.-J. Luan, L. Eloy, P. Bedossa, and J.-F. Bach. 1994. "Genetic Analysis of Immune Dysfunction in Non-obese Diabetic (NOD) Mice: Mapping a Susceptibility Locus Close to the *Bcl-2* Gene Correlates with Increased Resistance of the NOD T cells to Apoptosis Induction." *European Journal of Immunology.* 24: 380-384.

Gerosa, F., C. Paganin, D. Peritt, F. Paiola, M. T. Scupoli, M. Aste-Amezaga, I. Frank, and G. Trinchieri. 1996. "Interleukin-12 Primes Human CD4 and CD8 T cell Clones for High Production of Both Interferon-γ and Interleukin-10." *Journal of Experimental Medicine.* 183: 2559-2569.

Gill, B.M., H. Nishikata, G. Chan, T. L. Delovitch, and A. Ochi. 1994. "Fas Antigen and Sphingomyelin-Ceramide Turnover-Mediated Signaling: Role in Life and Death of T Lymphocytes." p.113-126. in "Apoptosis in Immunity"; dedicated volume of *Immunological Reviews*; G. Moller; ed. 142: December.

Giordano, C., R. DeMaria, M. Todaro, G. Stassi, A. Mattina, P. Richiusa, G. Galluzzo, F. Panto, and A. Galluzzo. 1993. "Study of T-Cell Activation in Type I Diabetic Patients and Pre-Type I Diabetic Subjects by Cytometric Analysis: Antigen Expression Defect in vitro." *Journal of Clinical Immunology.* 13(1): 68-78.

Goetzl, E. J., S. An, and L. Zeng. 1995. "Specific Suppression by Prostaglandin E2 of Activation-Induced Apoptosis of Human CD4+CD8+ T Lymphoblasts." *Journal of Immunology.* 154: 1041-1047.

Goetzl, E. J., M. J. Banda, and D. Leppert. 1996. "Matrix Metalloproteinases in Immunity." *Journal of Immunology.* 156: 1-4.

Gold, R., M. Schmied, G. Giegerich, H. Breitschopf, H. P. Hartung, K. V. Toyka, and H. Lassmann. 1994. "Differentiation between Cellular Apoptosis

and Necrosis by the Combined Use of in situ Tailing and Nick Translation Techniques." *Laboratory Investigation.* 71(2): 219-225.

Gonzalo, J.A., E. Baixeras, A. Gonzalez-Garcia, A. George-Chandy, N. Van Rooijen, C. Martinez-A., and G. Kroemer. 1994. "Differential in vivo Effects of a Superantigen and an Antibody Targeted to the Same T Cell Receptor." *Journal of Immunology.* 152: 1597-1608.

Goppelt-Struebe, M. 1995. "Regulation of Prostaglandin Endoperoxide Synthase (Cyclooxygenase) Isozyme Expression." *Prostaglandins, Leukotrienes, and Essential Fatty Acids.* 52: 213-222.

Groux, H., G. Monte, B. Plouvier, A. Capron, and J. C. Ameisen. 1993. "CD3-mediated Apoptosis of Human Medullary Thymocytes and Activated Peripheral T cells: Respective Roles of Interleukin-1, Interleukin-2, Interferon-γ, and Accessory Cells." *European Journal of Immunology.* 23: 1623-1629.

Gulbins, E., R. Bissonnette, A. Mahboubi, S. Martin, W. Nishioka, T. Brunner, G. Baier, G. Baier-Bitterlich, C. Byrd, F. Lang, R. Kolesnick, A. Altman, and D. Green. 1995. "FAS-Induced Apoptosis Is Mediated Via a Ceramide-Initiated RAS Signaling Pathway." *Immunity.* 2:341-351.

Hannun, Y. A. 1994. "The Sphingomyelin Cycle and the Second Messenger Function of Ceramide." *Journal of Biological Chemistry.* 269(5): 3125-3128.

Hannun, Y. A. 1997. "Functions of Ceramide in Coordinating Cellular Responses to Stress." *Science.* 274: 1855-1859.

Hannun, Y. A. and L. M. Obeid. 1995. "Ceramide: An Intracellular Signal for Apoptosis." *Trends in Biological Sciences.* 20(2): 73-77.

Holter, W., A. M. Spiegel, B. H. Howard, S. Weber, and M. R. Brann. 1991. "Expression of GTP-Binding Proteins and Prostaglandin E2 Receptors During Human T Cell Activation." *Cellular Immunology.* 134: 287-295.

Howie, S. E. M., D. J. Harrison, and A.H. Wyllie. 1994. "Lymphocyte Apoptosis--Mechanisms and Implications in Disease." p.141-156. in "Apoptosis in Immunity"; dedicated volume of *Immunological Reviews*; G. Moller; ed. 142: December.

Huang, L. and I. N. Crispe. 1993. "Superantigen-Driven Peripheral Deletion of T Cells." *Journal of Immunology.* 151(4):1844-1851.

Hyslop, S. and G. De Nucci. 1993. "Prostaglandin Biosynthesis in the Microcirculation: Regulation by Endothelial and Non-endothelial Factors." *Prostaglandins, Leukotrienes, and Essential Fatty Acids.* 49: 723-760.

Ihm, S.-H. and J.-W. Yoon. 1990. "Studies on Autoimmunity for Initiation of β–Cell-Specific Cytotoxic Effectors and Insulitis in NOD Mice." *Diabetes.* 39: 1273-1278.

Isomaki, P., R. Luukkainen, R. Saario, P. Toivanen, and J. Punnonen. 1996. "Interleukin-10 Functions as an Antiinflammatory Cytokine in Rheumatoid Synovium." *Arthritis & Rheumatism.* 39(3): 386-395.

Jansen, A., F. Homo-Delarche, H. Hooijkaas, P. J. Leenen, M. Dardenne, and H. A. Drexhage. 1994. "Immunohistochemical Characterization of Monocytes-Macrophages and Dendritic Cells Involved in the Initiation of the Insulitis and β-Cell Destruction in the NOD Mice." *Diabetes.* 43: 667-674.

Jarvis, W.D., F. A. Fornari, Jr., J. L. Browning, D. A. Gewirtz, R. N. Kolesnick, and S. Grant. 1994a. "Attenuation of Ceramide-induced Apoptosis by Diglyceride in Human Myeloid Leukemia Cells." *Journal of Biological Chemistry.* 269(50): 31685-31692.

Jarvis, W.D., R. N. Kolesnick, R.A. Fornari, R. S. Traylor, D. A. Gewirtz, and S. Grant. 1994b. "Induction of Apoptotic DNA Damage and Cell Death by Activation of the Sphingomyelin Pathway." *Proceedings of the National Academy of Sciences USA.* 91:73-77.

Jayadev, S. C. M. Linardic, and Y. A. Hannun. 1994. " Identification of Arachidonic Acid as a Mediator of Sphingomyelin Hydrolysis in Response to Tumor Necrosis Factor α." *Journal of Biological Chemistry.* 269(8): 5757-5763.

Jones, L. A., L. T. Chin, D. L. Longo, and A. M. Kruisbeek. 1990. "Peripheral Clonal Elimination of Functional T Cells." *Science.* 250: 1726-1729.

Katz, J. D., C. Benoist, and D. Mathis. 1995. "T Helper Cell Subsets in Insulin-Dependent Diabetes." *Science.* 268: 1185-1188.

Kawabe, Y. and A. Ochi. 1991. "Programmed Cell Death and Extrathymic Reduction of Vβ8+ CD4+ T Cells in Mice Tolerant to *Staphylococcus aureus* enterotoxin B." *Nature.* 349: 245-248.

Kayagaki, N., A. Kawasaki, T. Ebata, H. Ohmoto, S. Ikeda, S. Inoue, K. Yoshino, K. Okumura, and H. Yagita. "Metalloproteinase-mediated Release of Human *Fas* Ligand." *Journal of Experimental Medicine.* 182: 177-1783.

Kim, M-Y., C. Linardic, L. Obeid, and Y. Hannun. 1991. "Identification of Sphingomyelin Turnover as an Effector Mechanism for the Action of Tumor Necrosis Factor α and γ-Interferon." *Journal of Biological Chemistry.* 266: 484-489.

Kinoshita, T., T. Yokata, K. Araj, and A. Miyajima. 1995. "Suppression of Apoptotic Death in Hematopoietic Cells by Signaling through the IL-3/GM-CSF Receptors." *EMBO Journal.* 14(2):256-275.

Kishimoto, H., C. D. Surh, and J. Sprent. 1995. "Upregulation of Surface Markers on Dying Thymocytes." *Journal of Experimental Medicine.* 181: 649-655.

Kneitz, B., T. Herrmann, S. Yonehara, and A. Schimpl. 1995. "Normal Clonal Expansion but Impaired *Fas*-Mediated Cell Death and Anergy Induction in Interleukin-2-Deficient Mice." *European Journal of Immunology.* 25:2572-2577.

Kosaka, T., A. Miyata, H. Ihara, S. Hara, T. Sugimoto, O. Takeda, E. Takahashi, and T. Tanabe. 1994. "Characterization of the Human Gene (PGS2) Encoding Prostaglandin-Endoperoxide Synthase 2." *European Journal of Biochemistry.* 221: 889-897.

Krammer, P. H., J. Dhein, H. Walczak, I. Behrmann, S. Mariani, B. Matiba, M. Fath, P.T. Daniel, E. Knipping, M. O. Westendorp, K. Stricker, C. Baumler, S. Hellbardt, M. Germer, M. E. Peter, and K.-M. Debatin. 1994. "The Role of APO-1-Mediated Apoptosis in the Immune System." in "Apoptosis in Immunity";dedicated volume of *Immunological Reviews*; G. Moller; ed. 142: December.

Lang, R. A. and J. M. Bishop. 1993. "Macrophages Are Required for Cell Death and Tissue Remodeling in the Developing Mouse Eye." *Cell.* 74: 453-462.

Langenbach, R., S. G. Morham, H. G. Tiano, C. D. Loftin, B. I Ghanayem, P. C. Chulada, J.F. Mahler, C. A. Lee, E. H. Goulding, K. D. Kluckman, H.S. Kim, and O. Smithies. 1995. "Prostaglandin Synthase 1 Gene Disruption in Mice Reduces Arachidonic Acid-Induced Inflammation and Indomethacin-Induced Gastric Ulceration." *Cell.* 83: 483-492.

Lee, K.-U., K. Amano, and J.-W. Yoon. 1988. "Evidence for Initial Involvement of Macrophage in Development of Insulitis in NOD Mice." *Diabetes.* 37: 989-991.

Lee, M.-S., R. Mueller, L. S. Wicker, L. B. Peterson, and N. Sarvetnick. 1996. "IL-10 is Necessary and Sufficient for Autoimmune Diabetes in Conjunction with NOD MHC Homozygosity." *Journal of Experimental Medicine.* 183: 2663-2668.

Lenardo, M. J. 1991. "Interleukin-2 Programs Mouse ab T Lymphocytes for Apoptosis." *Nature.* 353: 858-861.

Leslie, C. A. and D. P. Dubey. 1994. "Increased PGE2 from Human Monocytes Isolated in the Luteal phase of the Menstrual Cycle. Implications for Immunity?" *Prostaglandins.* 47:41-54.

Liu,Y. and C. A. Janeway,Jr. 1990. "Interferon γ Plays a Critical Role in Induced Cell Death of Effector T Cell: A possible Third Mechanism of Self-tolerance." *Journal of Experimental Medicine.* 172: 1735-1739.

Lu, X., W. Xie, D. Reed, W. S. Bradshaw, and D. L. Simmons. 1995. "Nonsteroid Antiinflammatory Drugs Cause Apoptosis and Induce Cyclooxygenases in Chicken Embryo Fibroblasts." *Proceedings of the National Academy of Sciences.* 92: 7961-7965.

Ludviksson, B. R., B. Gray, W. Strober, and R. O. Ehrhardt. 1997. "Dysregulated Intrathymic Development in the IL-2-Dificient Mouse Leads to Colitis-Inducing Thymocytes." *Journal of Immunology.* 158: 104-111.

Maclaren, N. K., S. W. Huang and J. Fogh. 1975. "Antibody to cultured human insulinoma cells in insulin-dependent diabetes." *Lancet.* 1: 997.

Majno, G. and I. Joris. 1995. "Apoptosis, Oncosis, and Necrosis." American *Journal of Pathology.* 146(1): 3-15.

Mariani, S. M., B. Matiba, C. Baumler, and P. H. Krammer. 1995. "Regulation of Cell Surface APO-1/Fas (CD95) Ligand Expression by Metalloproteases." *European Journal of Immunology.* 25: 2303-2307.

Martin, S. J., D. R. Green, and T. G. Cotter. 1994. "Dicing with Death: Dissecting the Components of the Apoptosis Machinery." *Trends in Biological Sciences.* January: 26-30.

Mauel, J., A. Ransijn, S. B. Corradin, and Y. Buchmuller-Rouiller. 1995. "Effects of PGE2 and of Agents that Raise cAMP Levels on Macrophage Activation Induced by IFN-γ and TNF-α." *Journal of Leukocyte Biology.* 58: 217-224.

McConkey, D. J., P. Nicotera, and S. Orrenius. 1994. " Signaling and Chromatin Fragmentation in Thymocyte Apoptosis." in "Apoptosis in Immunity"; dedicated volume of *Immunological Reviews*; G. Moller; ed. 142: December.

Meisel, C., K. Vogt, C. Platzer, F. Randow, C. Liebenthal, and H.-D. Volk. 1996. "Differential Regulation of Monocytic Tumor Necrosis Factor-α and Interleukin-10 Expression." *European Journal of Immunology.* 26: 1580-1586.

Mertz, P. M., D. L. DeWitt, W. G. Stetler-Stevenson, and L. M. Wahl. 1994. "Interleukin 10 Suppression Monocyte Prostaglandin H Synthase-2." *Journal of Biological Chemistry.* 269(33): 21322-21329.

Miethke, T., R. Vabulas, R. Bittlingmaier, K. Heeg, and H. Wagner. 1996. "Mechanisms of Peripheral T Cell Deletion: Anergized T cells are Fas Resistant but Undergo Proliferation-associated Apoptosis." *European Journal of Immunology.* 26: 1459-1467.

Minakuchi, R., M.C. Wacholtz, L. S. Davis, and P. E. Lipsky. 1990. "Delineation of the Mechanism of Inhibition of Human T Cell Activation by PGE2." *Journal of Immunology.* 145(8): 2616-2625.

Morham, S. G., R. Langenbach, C. D. Loftin, H. F. Tiano, N. Vouloumanos, J. C. Jennette, J. F. Mahler, K. D. Kluckman, A. Ledford, C. A. Lee, and O. Smithies. 1995. "Prostaglandin Synthase 2 Gene Disruption Causes Sever Renal Pathology in the Mouse." *Cell.* 83: 473-482.

Morita, I., M. Schindler, M. K. Regier, J. C. Otto, T. Hori, D. L. DeWitt, and W. L. Smith. 1995. "Different Intracellular Locations for Prostaglandin Endoperoxide H Synthase-1 and -2." *Journal of Biological Chemistry.* 270(18): 10902-10908.

Mountz, J. D., J. Wu, J. Cheng, and T. Zhou. 1994. "Autoimmune Disease: A Problem of Defective Apoptosis." *Arthritis & Rheumatism.* 37(10): 1415-1420.

Nagata, S. and P. Golstein. 1995. "The *Fas* Death Factor." *Science.* 267: 1449-1455.

Niiro, H., T. Otsuka, T. Tanabe, S. Hara, S. Kuga, Y. Nemoto, Y. Tanaka, H. Nakashima, S. Kitajima, M. Abe, and Y. Niho. 1995. "Inhibition by Interleukin-10 of Inducible Cyclooxygenase Expression in Lipopolysaccharide-Stimulated Monocytes: Its Underlying Mechanism in Comparison with Interleukin-4." *Blood.* 85(12): 3736-3745.

Nishimura, Y., A. Ishii, Y. Kobayashi, Y. Yamasaki, and S. Yonehara. 1995. "Expression and Function of Mouse *Fas* Antigen on Immature and Mature T cells." *Journal of Immunology.* 154: 4395-4403.

Obeid, L. M., C. M. Linardic, L. A. Karolak, and Y. A. Hannun. 1993. "Programmed Cell Death Induced by Ceramide." *Science.* 259:1769-1771.

Owen-Schaub, L. B., S. Yonehara, W. L. Crump III, and E. A. Grimm. 1992. "DNA Fragmentation and Cell Death Is Selectively Triggered in Activated Human Lymphocytes by *Fas* Antigen Engagement." *Cellular Immunology.* 140: 197-205.

Paliogianni, F., R. L. Kincaid, and D. T. Boumpas. 1993. " Prostaglandin E2 and Other Cyclic AMP Elevating Agents Inhibit Interleukin 2 Gene Transcription by Counteracting Calcineurin-dependent Pathways." *Journal of Experimental Medicine.* 178: 1813-1817.

Parijs, L. V., A. Biuckians, A. Ibragimov, F. W. Alt, D. M. Willerford, and A. K. Abbas. 1997. "Functional Responses and Apoptosis of CD25(IL2Rα)-Deficient T cells Expressing a Transgenic Antigen Receptor." *Journal of Immunology.* 158: 3738-3745.

Pastor, M. I., K. Reif, D. Cantrell. 1995. "The Regulation and Function of *p21ras* during T-cell Activation and growth." *Immunology Today.* 16(3):158-164.

Podack, E.R. 1995. " Functional Significance of Two Cytolytic Pathways of Cytotoxic T Lymphocytes." *Journal of Leukocyte Biology.* 57: 548-552.

Prescott, S. M. and R. L. White. 1996. "Self-Promotion? Intimate Connections Between APC and Prostaglandin H Synthase-2." *Cell.* 87:783-786.

Reddy, S. T., and H. R. Herschman. 1994. "Ligand-induced Prostaglandin Synthesis Requires Expression of the TIS10/PGS-2 Prostaglandin Synthase Gene in Murine Fibroblasts and Macrophages." *Journal of Biological Chemistry.* 269(22): 15473-15480

Riese, J., T. Hoff, A. Nordhoff, D.L. DeWitt, K. Resch, and W. Kaever. 1994. "Transient Expression of Prostaglandin Endoperoxide Synthase-2

during Mouse Macrophage Activation." *Journal of Leukocyte Biology.* 55: 476-482.

Ristimaki, A. S. Garfinkel, J. Wessendorf, T. Maciag, and T. Hla. 1994. "Induction of Cyclooxygenase-2 by Interleukin 1-α." *Journal of Biological Chemistry.* 269(16): 11769-11775.

Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. "Immunologic Self-Tolerance Maintained by Activated T Cells Expressing IL-2 Receptor α-Chains (CD25)." *Journal of Immunology.* 155: 1151-1164.

Segal, B. M., D. M. Klinman, and E. M. Shevach. 1997. "Microbial Products Induce Autoimmune Disease by an IL-12-Dependent Pathway." *Journal of Immunology.* 158: 5087-5090.

Schorle, H., T. Holtschke, T. Hunig, A. Schimpl, and I. Horak. 1991. "Development and Function of T cells in Mice Rendered Interleukin-2 Deficient by Gene Targeting." *Nature.* 352: 621-624.

Schutze, S., T. Machleidt, and M. Kronke. 1994. " The Role of Diacylglycerol and Ceramide in Tumor Necrosis Factor and Interleukin-1 signal Transduction." *Journal of Leukocyte Biology.* 56: 533-541.

Serreze, D. V., K. Hamaguchi, and E. H. Leiter. 1989. "Immunostimulation Circumvents Diabetes in NOD/Lt Mice." *Journal of Autoimmunity.* 2: 759-776.

Serreze, D. V. and E. H. Leiter. 1988. "Defective Activation of T Suppressor Cell Function in Nonobese Diabetic Mice." *Journal of Immunology.* 140(11): 3801-3807.

Serreze, D.V., J. W. Graedeke, and E. H. Leiter. 1993. "Hematopoietic Stem-cell Defects Underlying Abnormal Macrophage Development and Maturation in NOD/Lt Mice: Defective Regulation of Cytokine Receptors and Protein Kinase C." *Proceedings of the National Academy of Sciences.* 90: 9625-9629.

Serreze, D. V. and E. H. Leiter. 1994. "Genetic and Pathogenic Basis of Autoimmune Diabetes in NOD Mice." *Current Opinion in Immunology.* 6: 900-906.

Sharfe, N., H. K. Dadi, M. Shahar, and C. M. Roifman. 1997. "Human Immune Disorder Arising from Mutation of the α chain of the Interleukin-2

Receptor." *Proceedings of the National Academy of Sciences.* 94: 3168-3171.

Sneller, M. C., S. E. Straus, E. S. Jaffe, J. S. Jaffe, T. A. Fleisher, M. Stetler-Stevenson, and W. Strober. 1992. " A Novel Lymphoproliferative/ Autoimmune Syndrome Resembling Murine *lpr/gld* Disease." *Journal of Clinical Investigation.* 90: 334-341.

Snijdewint, F. G. M., P. Kalinski, E. A. Wierenga, J. D. Bos, and M. L. Kapsenberg. 1993. "Prostaglandin E2 Differentially Modulates Cytokine Secretion Profiles of Human T Helper Lymphocytes." *Journal of Immunology.* 150: 5321-5329.

Spittler, A., C. Schiller, M. Willheim, C. Tempfer, S. Winkler and G. Boltz-Nitulescu. 1995. "IL-10 Augments CD23 Expression on U937 Cells and Down-regulates IL-4-driven CD23 Expression on Cultured Human Blood Monocytes: Effects of IL-10 and Other Cytokines on Cell Phenotype and Phagocytosis." *Immunology.* 85: 311-317.

Squier, M. K. T., A. J. Sehnert, and J. J. Cohen. 1995. "Apoptosis in leukocytes." *Journal of Leukocyte Biology.* 57: 2-10.

Steller, H. 1995. "Mechanisms and Genes of Cellular Suicide." *Science.* 267: 1445-1449.

Strassmann, G., V. Patil-Koota, F. Finkelman, M. Fong, and T. Kambayashi. 1994. "Evidence for the Involvement of Interleukin 10 in the Macrophages by Prostaglandin E2." *Journal of Experimental Medicine.* 180: 2365-2370.

Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. " Molecular Cloning and Expression of the *Fas* Ligand, a Novel Member of the Tumor Necrosis Factor Family." *Cell.* 75: 1169-1178.

Sunderkotter, C., K. Steinbrink, M. Goebeler, R. Bhardwaj, C. Sorg. 1994. "Macrophages and Angiogenesis." *Journal of Leukocyte Biology.* 55: 410-422. 1994.

Sweet, M. J., and D. A. Hume. 1996. Endotoxin signal transduction in macrophages. *J Leukocyte Biology.* 60: 8-26.

Taguchi, O. and T. Takahashi. 1996. "Administration of Anti-interleukin-2 receptor α antibody in vivo induces localized autoimmune disease." *European Journal of Immunology.* 26: 1608-1612.

Takenaka, H. S. Maruo, N. Yamamoto, M. Wysocka, S. Onon, M. Kobayashi, H. Yagita, K. Okumura, T. Hamaoka, G. Trinchieri, and H. Fujiwara. 1997. "Regulation of T cell dependent and -independent IL-12 production by the three Th2-type cytokines IL-10, IL-6, and IL-4." *Journal of Leukocyte Biology.* 61: 80-87.

Thompson, C. B. 1995. "Apoptosis in the Pathogenesis and Treatment of Disease." *Science.* 267: 1456-1462.

Tian,Q., J.-L. Taupin, S. Elledge, M. Robertson, and P. Anderson. 1995. "Fas-activated Serine/Threonine Kinase (FAST) Phosphorylates TIA-1 during Fas-mediated Apoptosis." *Journal of Experimental Medicine.* 182: 865-874.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy Sciences.* 76(9):4350-4354.

Tsujii, M. and R.N. DuBois. 1995. "Alterations in Cellular Adhesion and Apoptosis in Epithelial Cells Overexpressing Prostaglandin Endoperoxide Synthase 2." *Cell.* 83: 493-501.

Ucker, D.S., J. Meyers, and P. S. Obermiller. 1993. "Activation-Driven T Cell Death; II. Quantitative Differences Alone Distinguish Stimuli Triggering Nontransformed T Cell Proliferation or Death." *Journal of Immunology.* 149: 1583-1592.

Ucker, D.S., L. D. Hebshi, J. F. Blomquist, and B. E. Torbett. 1994. "Physiological T-Cell Death: Susceptibility is Modulated by Activation, Aging and Transformation, but the Mechanism is Constant." in "Apoptosis in Immunity"; dedicated volume of *Immunological Reviews*; G. Moller; ed. 142: December.

van der Pouw Kraan, T. C. T. M., L. C. M. Boeije, R. J.T. Smeek, J. Wijdenes, and L. A. Aarden. 1995. "Prostaglandin E2 is a Potent Inhibitor of Human Interleukin 12 Production." *Journal of Experimental Medicine.* 181: 775-779.

Wang, R., A. M. Rogers, B.J. Rush, and J. H. Russell. 1996. "Induction of Sensitivity to Activation-induced Death in Primary CD4+ Cells: a Role for Interleukin-2 in the Negative Regulation of Responses by Mature CD4+ T Cells." *European Journal of Immunology.* 26: 2263-2270.

Watanabe-Fukunaga, R., C. I. Brannan, N. G. Copeland, N. A. Jenkins, and S. Nagata. 1992. "Lymphoproliferation Disorder in Mice Explained by Defects in *Fas* Antigen that Mediates Apoptosis." *Nature.* 356:314-317.

Watson, J. and E. S. Wijelath. 1990. "Interleukin-one Induced Arachidonic Acid Turnover in Macrophages." *Autoimmunity.* 8: 71-76.

Webb, S., C. Morris, and J. Sprent. 1990. "Extrathymic Tolerance of Mature T Cells: Clonal Elimination as a Consequence of Immunity." *Cell.* 63: 1249-1256.

Weber, G. F., S. Abromson-Leeman, and H. Cantor. 1995. "A Signaling Pathway Coupled to T Cell Receptor Ligation by MMTV Superantigen Leading to Transient Activation and Programmed Cell Death." *Immunity.* 2: 363-372.

Wicker, L. S., J.A. Todd, and L. B. Peterson. 1995. "Genetic Control of Autoimmune Diabetes in the NOD Mouse." *Annual Reviews in Immunology.* 13: 179-200.

Winter, W. E., T. Chihara, and D. Schatz. 1993. "The Genetics of Autoimmune Diabetes." *American Journal of Diabetes Consultants.* 147: 1282-1290.

Wu, T., S. J. Levine, M. G. Lawrence, C. Logun, C. W. Angus, and J. H. Shelhamer. 1994. "Interferon-g Induces the Synthesis and Activation of Cytosolic Phospholipase A2." *Journal of Clinical Investigation.* 93: 571-577.

Xie, T. 1997. "The Role of Prostaglandin Synthase 2 (PGS2) in the Immunopathogensis of NOD Diabetes". Doctoral Dissertation. University of Florida. College of Medicine.

Yui, M., K. Muralidharan, B. Moreno-Altamirano, G. Perrin, K. Chestnut, and E. K. Wakeland. 1996. "Production of Congenic Mouse Strains Carrying NOD-derived Diabetogenic Genetic Intervals: An Approach for the Genetic Dissection of Complex Traits." *Mammalian Genome.* 7(5): 331-334.

Zhu, L. and C. Anasetti. 1995. "Cell Cycle Control of Apoptosis in Human Leukemic T Cells." *Journal of Immunology.* 154: 192-200.

BIOGRAPHICAL SKETCH

Sally A. Litherland was born in Melbourne, Florida. She received her Bachelor of Science in Food and Consumer Protection/Food Science from the University of Florida in 1981, a Masters of Science in Nutritional Biochemistry from University of Florida in 1983, and a Masters of Science in Molecular Biology from Cornell University Medical College in 1990. She worked as the Senior Mission Support Scientist and Deputy Manager of Mission Operations for the Kennedy Space Center NASA Life Sciences contractor from 1990 to 1994. She has been a Science Consultant and Educational Public Affairs Lecturer for NASA from 1990 to the present. Upon completion of her doctoral studies, she will return to Florida's Space Coast area to pursue a career in education and academic research.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael J. Clare-Salzler, Chair
Associate Professor of Pathology,
Immunology, and Laboratory
Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ammon B. Peck
Professor of Pathology, Immunology,
And Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edward Wakeland
Professor of Pathology, Immunology,
And Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Charles Allen
Professor of Biochemistry and
Molecular Biology

This dissertation was submitted to the Graduate Faculty of the Department of Pathology, Immunology,and Laboratory Medicine, College of Medicine, and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1997

Dean, College of Medicine

Dean, Graduate School